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Culture of Fresh Water Algae in Enriched Natural Sea Water

By

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(Received July 15, 1960)

While screening a large number of unicellular green algae for their ability to absorb certain ions from sea water, it was observed that many well known fresh water species grew rapidly in high concentrations of sea water if certain mineral nutrients normally absent, or present in low concentrations, were supplied. In view of the widespread interest in the mass culture of these algae and the limitations imposed by the use of fresh water in mass cultures, more detailed tests of the salt tolerance of a number of common fresh water algae were conducted.

Materials and Methods

The data presented in Table 1 were obtained culturing the algae in liquid media in 3 ml. glass planchets placed in Petri dishes. Conditions of culture were: illuminance, 250 foot candles ("cool white" fluorescent light); temperature, 22°C. The planchets were inoculated with a standardized bacteriological loop from liquid non-saline starter cultures to assure uniformity of inoculum.

The agar plate auxanograph tests were made in Petri dishes on solid media prepared by mixing the sea water medium with 1 per cent agar. Conditions of culture are given above.

The data presented in Figure 1 represent the growth obtained in test tube cultures through which air of 3 per cent carbon dioxide was bubbled. Conditions of culture were: illuminance, 800 foot candles ("cool white" fluorescent light supplied from sources on opposite sides of the culture vessels); temperature, 25°C.

The sea water medium used in all experiments consisted of 1 g. NaNO_3 , 0.11 g. KH_2PO_4 , and trace elements, per liter of natural sea water (salinity, 31.2 ‰). The

trace elements Fe, Mn, Ca, Co, Zn, and Cu were supplied as salts of ethylenediamine-tetraacetic acid in the quantities recommended by Krauss (1955).

The non-saline medium used in all experiments consisted of 1 g. KNO_3 , 0.25 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.11 g. KH_2PO_4 , and trace elements (as above) per liter of distilled water (specific conductance, 1.51×10^{-6} mhos).

Dilutions of sea water medium were made with non-saline medium to avoid dilution of essential nutrients. All media were adjusted to pH 6.5–7.0. Media were sterilized by filtration, and aseptic technique was used throughout.

Growth, as presented in Table 1 and Figure 1, was measured as the optical density of the culture at 560 m μ . In the auxanograph tests, growth was estimated visually by comparison with control preparations.

Results and Discussion

Table 1 presents the relationship between growth and the concentration of sea water medium for 13 fresh water species of unicellular green algae. To conserve space, data for the intermediate concentrations of 10, 30, 50, 70, and 90 per cent sea water medium were omitted; however, notation is made in the table where these data become important. Incubation periods were chosen to compensate for inherent differences in the rate of growth of each species.

Response varied from tolerance of full strength sea water medium (*e.g.* *Chlorella*) to strong inhibition even at low concentration of sea water medium

Table 1. Growth of unicellular fresh water algae in different concentrations of sea water medium as indicated by the optical density of the culture. Numbers represent optical density of cultures read at 560 m μ . Values below .09 represent light absorption and scattering by precipitates and inoculum and no not indicate growth.

Species	Incubation Period (Days)	Percentage of sea water in culture medium					
		0	20	40	60	80	100
<i>Ankistrodesmus braunii</i>	5	1.35	.93	.12	.09	.06	.06
<i>Chlamydomonas eugametos</i>	5	.99	1.35	1.17	1.02 ¹	.12	.12
— <i>intermedia</i>	4	1.14 ¹	.15	.12	.09	.03	.03
— <i>reinhardtii</i>	5	1.29	.75 ¹	—	.09	.06	.06
<i>Chlorella ellipsoidea</i>	12	1.26	.93	.45	.12	.09	.06
— <i>pyrenoidosa</i> (Emerson strain) ..	8	1.47	1.05	1.11	1.11	1.02	.63
— <i>sp</i> 7-11-05 (Sorokin strain)	8	1.68	1.50	1.20	.75	.42	.30
<i>Coccomyxa simplex</i>	6	1.50	1.02	.87	.51	—	—
<i>Coelastrum proboscideum</i>	7	.66	.39	.09	.09	.09	.09
<i>Gyrorffiana humicola</i>	9	.96	1.29	1.11	.96	.67	.03
<i>Scenedesmus brasiliensis</i>	11	1.98	.99 ¹	.18	.15	.09	.09
— <i>obliquus</i> (Krauss strain)	10	.93	.78 ¹	.21	.09	.09	.09
— <i>quadricauda</i>	10	.93	.87	.50 ¹	.24	.03	.03

¹ Instances where significant growth occurred between this figure and the next higher concentration of sea water.

(e.g. *Chlamydomonas intermedia*, *Coelastrum proboscideum*). Several species grew in concentrations of sea water medium as high as 60 ‰ at rates equal to or closely approaching the rates on fresh water media. These data illustrate the suitability of enriched natural sea water for algal culture.

The influence of the osmotic shock, at the time of inoculation, upon the growth limits presented here was tested in the following way. Cells were grown at sea water concentrations just below the apparent upper limits shown in Table 1. They were then transferred to media of both higher and lower salinity levels and growth measured after an incubation period equal to that of the experiments presented in Table 1. Of nine species so tested only one, *Chlamydomonas eugametos* showed growth at higher salinities. This test also indicated that adaptation to the physiological stress of high salinity is not readily induced in these organisms by preliminary culture at sub-limiting salinity levels.

Although the data in Table 1 serve well to illustrate the wide range of salt tolerance in these algae, the limits of growth recorded here must not be taken as the absolute limits. Modified environmental conditions may raise or lower these limits considerably. Figure 1 presents graphically the time-course of growth of *Scenedesmus obliquus* at 4 salinity levels when the culture was agitated in the presence of 3 per cent carbon dioxide in the air at a light intensity ca. 3 times higher than used in the planchet experiments. Not only is the rate of growth greatly increased for all media, but the range of tolerance is nearly doubled. The physiological significance of these environmental factors is under investigation.

The possibility of altering salt tolerance by adding potential metabolites, vitamins, mineral nutrients, and crude extracts to the culture medium was exploited using an agar plate auxanographic technique with *Scenedesmus obliquus*, and *Coelastrum proboscideum*. Algal suspensions were sprayed aseptically onto the surface of a solid sea water medium containing 1 per cent agar. The salinity of the culture media was just high enough to inhibit the growth of these organisms, as shown in Table 1. A highly uniform dispersal of the cells was obtained in this way. Crystals or droplets of the test substances were placed directly on the agar. Among the compounds tested were soil extract, yeast extract, veal extract, casein hydrolysate, gelatin, glucose, pyruvic, succinic, and acetic acids, ethylenediaminetetraacetic acid, and 9- γ -fluorohydrocortisone. With the exception of gelatin, none of the materials tested reduced the inhibition of colony development caused by high salinity. In contrast, Ingram (1957) reviewed a number of reports of successful increase of the salt tolerance of halophilic yeasts and bacteria resulting from the addition of vitamins and metabolites.

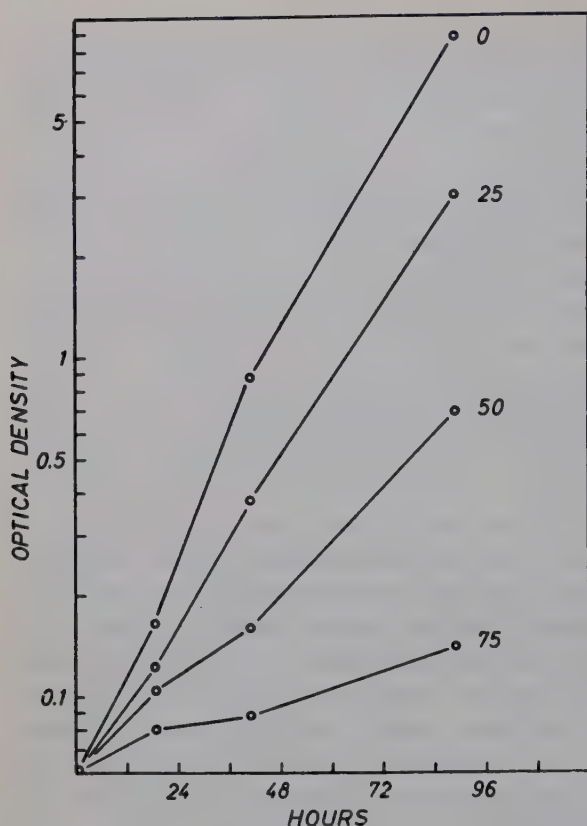


Figure 1. The growth of *Scenedesmus obliquus* at four salinity levels (0, 25, 50, and 75 per cent sea water medium). Illuminance 800 f.c., aeration with 3 per cent CO₂-in-air, temperature 25°C.

Commercially purified bacteriological gelatins from two sources permitted normal green colonies to develop in a one inch wide zone around the point of application. This activity of gelatin was confirmed in repeated tests with both algae species. All colonies outside of the zone of influence of gelatin were small and yellow or bleached white. The possibility that the gelatin was contaminated with, or was metabolized to yield, an active compound is unlikely for no activity was detected in similar tests of casein hydrolysate, egg albumin, or crude meat extracts.

Stuart (1940) reported that gelatin stimulated the growth of a halophilic bacterium (*Sarcina* sp.) cultured on highly saline media. This stimulation was also not explained in terms of a potential nutritive function of the gelatin. The present work provides no satisfactory explanation of this effect.

The exhibition of salt tolerance by these common fresh water or terrestrial species should not be too surprising. At least two of the genera tested (*Chla-*

mydomonas and *Chlorella*) are known to have marine species. *Scenedesmus*, *Coelastrum* and *Ankistrodesmus*, are in the same taxonomic order as *Chlorella*. The brine algae *Dunaliella*, *Platmonas*, and *Stephanoptera* (Gibor 1956) are closely related to *Chlamydomonas*.

It is interesting to raise the question of the nature of the phylogenetic origin of these apparently related organisms inhabiting distinct and probably mutually exclusive habitats. If one group is ancestral to the other, one might expect to find residual physiological characteristics in the derived group, characteristics which at one time may have permitted the successful reproduction of the species in a very different environment. The present work shows that a number of fresh water algae are able to grow rapidly in saline media. It is also known that certain of these algae can exist in fresh water only with the aid of a complex contractile vacuole system which permits these organisms to maintain an internal osmotic concentration higher than that of the external medium. (Krogh 1939, Guillard 1954). It may be that the ability to grow in saline media is the ancestral characteristic and that the ability to cope with the non-saline environment is a modification of the ancestral type.

Summary

Thirteen unicellular, green, fresh water algae were cultured in a series of dilutions of enriched sea water. Response varied from tolerance of full strength sea water medium to strong inhibition of growth by even low concentrations of sea water medium. Limits of tolerance could be altered by changing the environmental conditions, but not by the addition of any of a variety of organic substances to the culture medium. The influence of the osmotic shock experienced when cells grown in non-saline media were inoculated into saline media was found not to alter the results of these tests.

These tests also show that when natural sea water is enriched with the proper concentrations of nitrate, phosphate and iron salts, and when the pH is reduced to prevent precipitation of nutrients, it provides a medium suitable for algal culture. The aeration of enriched natural sea water or brackish water with a mixture of carbon dioxide-in-air should make possible high yields of *Chlorella pyrenoidosa*, *Scenedesmus obliquus* and other fresh water algae which have proved valuable in mass culture studies.

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Metabolic Processes Associated with Growth in Storage Tissue Disks

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Criteria commonly taken to establish that growth is taking place include increases in the volume of the tissue and in the amount of some cell components especially protoplasmic protein. Judged by these criteria conflicting reports have appeared concerning the occurrence of growth in storage tissue disks maintained in salt or aqueous media. Steward has consistently stressed that the capacity for renewed growth as evidenced by protein synthesis is a *sine qua non* for salt accumulation by potato disks, the metabolic state of which is an unstable equilibrium quite different from that of dormant storage tissue (see Steward and Millar 1954 for a summary of his earlier work). On the other hand Sutcliffe (1954) reported that red beet disks showed no increase in volume and little overall synthesis of protein after 48 hours. On these grounds he concluded that the disks consisted of mature cells, therefore affording a non-growing relatively stable system in which salt uptake was independent of net protein synthesis.

Synthesis of protein has been confirmed a number of times in potato slices (Mulder 1955, Calo and Varner 1957, Thimann and Loos 1957) and it has been reported in radish disks (Said and El-Shishiny 1947) but a systematic study of the occurrence and extent of protein synthesis in disks of a variety of storage tissues has not hitherto been undertaken. In this paper details are given of fresh weight changes and changes in some organic constituents of disks of four storage tissues maintained in running tap water. Taken in conjunction with changes in structural compounds already reported (Knight, Crooke, MacDonald and Shepherd, in press) a fairly complete picture is thus obtained of the extent of growth changes in disks under these conditions.

Methods

Disks from storage tissue of red beet (*Beta vulgaris* var. *rapa*), sugar beet (*B. vulgaris* var. *saccharifera*), carrot (*Daucus carota*), and swede (*Brassica napus*) were prepared and maintained in continuously changing tap water under controlled conditions of temperature and aeration as previously described (MacDonald and Knight 1958). Experiments were run at 25°C, 15°C and 2°C, a refrigeration coil being inserted in the washing unit for the low temperature experiments. The disk dimensions were generally 11 mm. × 1.0 mm. but the beets were occasionally sliced 0.5 mm. thick. Samples were withdrawn daily or at other suitable intervals.

The fresh weight was measured on a sample of not less than 50 disks. The disks were evenly blotted between filter paper and immediately weighed. The weight of water-insoluble material was obtained by boiling 30 disks in 60 ml. H₂O for 5 minutes and drying the residue overnight at 80°C. Nitrogen values were obtained from a 10 disk sample which was extracted in 2.5 % (w/v) trichloroacetic acid on a water bath at 100°C for 20 minutes. The extract was filtered and made up to volume. Soluble N was determined in the filtrate and insoluble N in the residue by the micro-Kjeldahl method using mercuric sulphate as a catalyst.

The free amino acid content of a 50 disk sample was determined by a method previously described (DeKock and Morrison 1958a). Briefly the disks were extracted with 70 % acetone in a Waring Blendor and the residue was further extracted with hot 0.01 N HCl. The extract was reduced in volume *in vacuo* and passed through a Zeo-karb 225 column. The amino acids retained were subsequently eluted with aqueous 2 N ammonia and the excess ammonia was evaporated off under reduced pressure. The total amino acids in the eluate were determined by decarboxylation of an aliquot with chloramine T in a Warburg apparatus (Kemble and MacPherson 1954).

The organic acid content of the same sample was estimated by passing the effluent from the Zeo-karb 225 column through an Amberlite IR4B column which absorbed the organic acids. These were subsequently eluted with aqueous N-NH₃ solution and concentrated to 10 ml. by evaporation on a water-bath. A quantitative separation of the organic acids was then achieved by fractionation on a silica gel column (DeKock and Morrison 1958 b).

Total soluble and insoluble phosphorus was determined by crushing 20 disks in a chilled mortar with ice-cold 10 % trichloroacetic acid. The extraction was continued for 15 minutes with 5 % TCA. The residue and supernatant were centrifuged at 7000 r.p.m. for 15 minutes when the supernatant was carefully decanted, the residue resuspended in 5 % TCA and again centrifuged. Supernatant and residue were transferred to silica crucibles and ashed overnight at 450°C. Phosphorus was then determined on the HCl extract of the ash by the hydrazine-molybdate method. Total phosphorus was also determined on boiled disks and it was found that this value roughly corresponded to the P present in the TCA residue.

Both the fresh weight and the dry weight of the disks change with time and consequently the weight of freshly cut disks is used as a basis of calculation, all measurements being calculated back to this initial fresh weight value.

Results

Changes in the fresh weight and dry weight of disks with time

Freshly cut disks immersed in water show an immediate increase in fresh weight and in carrot and beet water uptake continues for at least a week. In swede disks there is little further gain after the first 48 hours. The percentage increase in weight is shown in Figure 1.

Due to respiratory consumption of the carbohydrate reserves the dry weight of disks of each tissue falls linearly with time (Knight, *et al.*, in press) but if the disks are boiled in order to eliminate the contribution of the storage sugars to the dry weight, the weight of the water insoluble material in the disks shows a definite increase with time. Table 1 shows the absolute increases in the dry weight of boiled disks of 4 tissues maintained at 15°C and 25°C. Again the increase is continuous over a period of days with the exception of swede where there is little further change after 48 hours.

Changes in soluble and insoluble nitrogen

An overall increase in the total N content of beet and carrot disks washed in tap water has already been reported (MacDonald, DeKock and Knight 1960) and presumably results from the absorption of free nitrate from the tap water where it is present in a concentration of 0.1 ppm. The results

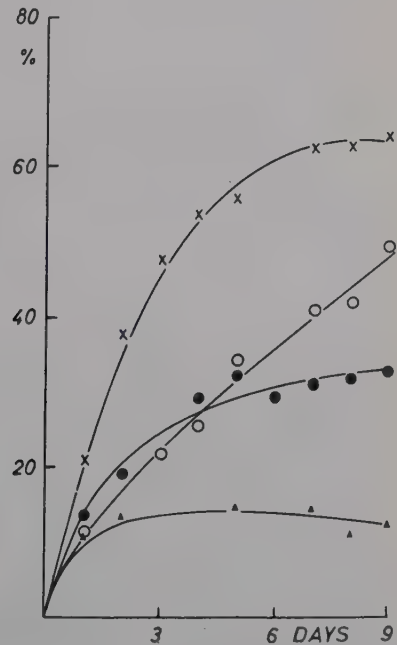


Figure 1. Percentage increase in fresh weight of disks during washing at 25°C. ×—× red beet, ○—○ sugar beet, ●—● carrot, ▲—▲ swede.

Table 1. *Changes in the content of water-insoluble material of disks washed at 15°C and 25°C i.e., dry weight expressed as µg./g. initial fresh weight of disks after boiling.*

Day	Sugar beet		Red beet		Carrot		Swede	
	15°C	25°C	15°C	25°C	15°C	25°C	15°C	25°C
0	42.6	41.4	28.9	34.1	44.0	44.0	35.3	30.0
1	50.6	48.1	34.7	45.9	48.1	51.7	35.9	31.6
2	56.5	54.0	—	51.5	50.3	52.8	41.8	36.4
4	57.5	60.4	40.4	57.3	52.9	54.0	40.6	35.2
5	—	—	42.8	59.7	54.2	55.8	39.1	36.7
6	62.8	72.8	44.8	58.1	55.4	53.4	42.6	35.5
8	68.9	79.3	48.8	—	55.5	—	41.5	—

obtained from soluble and insoluble N analyses of disks maintained at 25°C, 15°C and 2°C are depicted graphically in Figure 2 and it can be deduced from the graphs that the level of total N increases particularly in the beet. The most noticeable feature of the analyses is the very considerable increase in the insoluble N content of some tissues. In the beets this amounts to a 3-fold increase at 25°C and carrot is not far short of this level. Synthesis at 15°C is slower but a comparable level is eventually reached. In swede the overall changes are less and in all tissues synthesis is prevented at 2°C. For the most part changes in soluble N show a converse pattern to the insoluble changes although latterly the soluble N may also increase. Total N was separately determined and good agreement obtained between it and the sum of soluble and insoluble N. Evidence that the increase in insoluble N in the disks was due to the synthesis of protein was obtained by hydrolysing a sample of disks overnight with 6 N HCl in sealed glass bulbs at 80°C. The amino acid pattern developed from the hydrolysates of fresh disks and disks washed for varying periods showed no change in the nature or number of the acids present but a very pronounced intensification of the spots was evident with time.

Changes in insoluble nitrogen in disks in distilled water

The absorption of nitrate from tap water could obviously greatly influence the nitrogen metabolism of the disks and in order to determine whether the effect of tap water in general and nitrate in particular on protein synthesis was quantitative rather than qualitative, analyses were carried out on disks which had been washed in distilled water. It was found impractical to wash the disks continuously with fresh distilled water but in order to simulate the conditions of continuous washing a closed system was constructed in which distilled water from a 45 litre carboy was circulated by means of a pump to the 5 litre washing flask containing 200 disks. The distilled water in the carboy was changed twice a day. Red beet disks maintained under

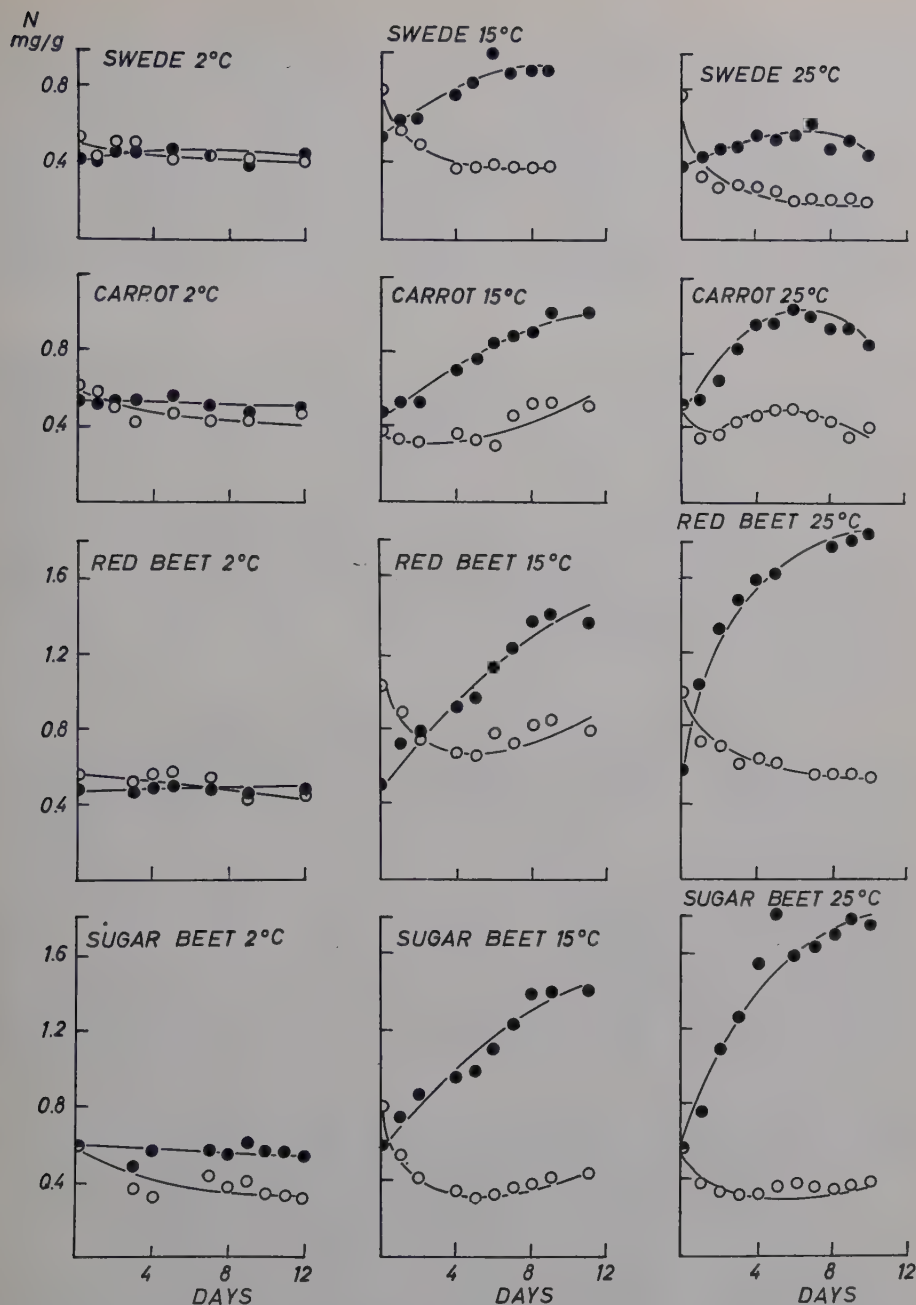


Figure 2. Changes in the content of soluble and insoluble nitrogen in disks washed at 2°C, 15°C and 25°C. ●—● insoluble N, ○—○ soluble N, expressed as mg N per g. fresh weight.

Table 2. *Changes in TCA soluble P and TCA insoluble P expressed as $\mu\text{g./g.}$ initial fresh weight. TCA insoluble P also expressed as percentage of Total P. Disks washed at 25°C.*

Day	Sugar beet			Red beet			Carrot			Swede		
	Sol.	Insol.	% Insol.	Sol.	Insol.	% Insol.	Sol.	Insol.	% Insol.	Sol.	Insol.	% Insol.
0	67.2	42.1	38.5	124.1	37.0	23.0	402.7	80.5	16.7	184.4	66.8	26.4
1	56.6	45.6	44.4	96.4	71.3	42.5	255.5	86.1	25.2	166.9	72.3	30.2
2	—	—	—	—	—	—	272.2	125.0	31.5	119.6	75.1	38.6
3	41.0	57.0	58.1	66.0	73.9	52.8	300.0	147.2	32.9	150.2	72.3	32.5
4	38.7	62.6	61.8	68.6	85.8	56.6	227.8	119.4	34.4	116.8	86.2	42.5
5	25.1	75.6	75.1	63.4	84.5	57.1	256.6	157.2	38.0	105.7	66.8	38.7

these conditions showed a fall in total N amounting to 10 % over an 8 day period at 25°C. Protein synthesis continued for 4 days during which time the protein content increased from 0.51 mg. N/g. fresh weight to 1.025 mg. N. Soluble N fell during this period and thereafter both fractions remained steady. Sugar beet and carrot disks also synthesised protein in distilled water and in sugar beet the conversion of soluble to insoluble N was shown to be temperature determined. Obviously then the effect of nitrate in the tap water was to promote protein synthesis to a level beyond that attainable with the nitrogen reserves present in the storage tissue.

Changes in soluble and insoluble phosphorus

As the disks age there is a decrease in the TCA soluble P and an increase in the TCA insoluble P indicating the conversion of soluble P to insoluble forms. These values along with the percentage of insoluble P in the disks are shown in Table 2. In swede disks the increase in insoluble P is small and appears to be confined almost entirely to the first 2 days but in the other tissues the content of insoluble P is doubled and the increase continues over several days. Further evidence of the increase in phosphorus associated with insoluble compounds was obtained by estimating the total phosphorus in boiled disks (Table 3). The results agreed with those obtained by TCA extrac-

Table 3. *Changes in the content of water insoluble P of disks washed at 15°C. Total P of boiled disks expressed as micrograms/g. initial fresh weight.*

Day	Sugar beet	Red beet	Carrot	Swede
0	42.5	42.4	85.2	86.4
1	54.2	44.6	93.3	76.8
2	66.1	54.9	105.6	95.4
4	81.7	57.5	113.1	89.1
5	74.7	64.5	121.6	86.4
6	91.5	66.5	115.0	91.7
7	89.4	68.8	—	82.9

Table 4. *Changes in the content of individual organic acids expressed as μ equiv./g. initial fresh weight in red beet disks washed at 25°C.*

Day	Fumaric	Succinic	Malonic	Oxalic	Malic	Citric
0	0.27	0.49	3.18	5.69	4.74	21.22
1	0.61	1.71	2.16	4.09	7.69	17.09
2	1.25	2.92	2.05	10.84	7.09	14.67
3	1.10	3.04	1.06	36.16	9.32	17.88
4	1.25	2.46	1.14	40.86	6.78	16.22
5	1.35	1.19	1.40	69.35	3.87	12.11
6	1.17	1.10	1.14	70.68	1.55	7.89

tion in that the greatest increase occurred in the beets while the increase in swede was small and not maintained.

Changes in organic and amino acids in beet tissue

The total organic acid content of red beet disks as revealed by titratable acidity increases with time. Separation of the individual acids of an extract showed that this increase was entirely due to the production of oxalic acid. The daily values recorded for the principal acids in red beet disks maintained at 25°C over a period of 6 days are enumerated in Table 4. Sugar beet disks gave similar results particularly as regards oxalate formation.

The total amino acid content, reflecting the decrease in soluble N in red beet disks at 25°C, falls rapidly during the earlier part of the washing period (Figure 3).

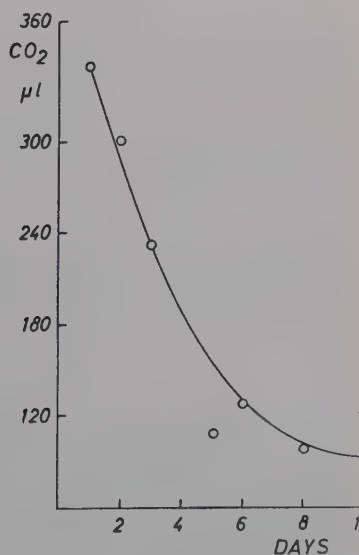


Figure 3. *Total amino acid content expressed as μ l CO₂ evolved/g. fresh weight of red beet disks washed at 25°C.*

Discussion

Slicing initiates in the metabolism of storage tissue a basic alteration most prominently expressed in an increased respiratory activity. So much at least is beyond dispute. However, as regards the nature and extent of growth phenomena — specifically cell enlargement, cell division and protein synthesis — occurring as a consequence of slicing, estimates have ranged from absent to conspicuous. The question is an important one in relation to the interpretation of the respiratory and other features of the disks' metabolism particularly salt absorption and accumulation.

Cell division and cell enlargement

The occurrence of cell division in potato disks was first reported by Steward, Wright and Berry (1932). Their findings were confirmed by Hackett and Thimann (1952) who observed marked division in disks maintained 8 days in distilled water although surprisingly Thimann and Loos (1957) following an identical procedure reported that no appreciable cell division occurred during that period. As regards other tissues Turner (1940) found no meristematic activity in carrot disks nor did Bennet-Clark and Bexon (1943) find any signs of cambial activity in red beet disks a finding with which Sutcliffe (1952) concurred. Microscopic examination of sections of disks has yielded evidence of cell divisions occurring in sugar beet disks (MacDonald 1955). Sinnott and Bloch (1941) maintain that meristematic activity may be induced in mature relatively large and highly vacuolated cells by wounding an adjacent region and the pattern they outline for division in such cells is identical with that observed in the cells of sugar beet disks and agrees with the description of nuclear changes in potato cells recorded by Steward *et al.* (1932). Although fresh weight changes of the order of those reported here for beet and carrot could conceivably be accounted for by cell enlargement alone it may be significant that swede disks which are without cambial initials show the smallest increase in volume. A period of increasing sensitivity of the respiration to cyanide has already been reported as suggesting the occurrence of cell division in beet disks (MacDonald 1959). All in all it would seem that growth by cell enlargement and cell division does occur in disks maintained in aqueous media.

Protein synthesis

Whether or not the increase in volume of the disks results from cell enlargement or division it is certainly accompanied by protein synthesis at the expense of soluble N reserves. Inspection of the preceding figures will

show that there is a positive correlation between growth (increase in fresh weight) and synthesis, red beet showing the maximum and swede the minimum development. Synthesis can also be positively correlated with the level of carbohydrate reserves held by the tissue since carbohydrate is most plentiful in the beet.

Hitherto the only positive findings of protein synthesis have been reported for radish (Said and El-Shishiny, 1947) and by several workers for potato. Thimann and Loos (1957) for example reported a 66 % increase in protein (from 0.85 mg. protein N/g. fresh weight to 1.41 mg.) in potato disks maintained in distilled water during 4 days. Under our somewhat vigorous conditions of aeration and circulation potato disks tend to disintegrate rather rapidly especially at 25°C and therefore potato has not been so intensively studied as other tissues but we have confirmed that synthesis does occur *e.g.* during a 4 day period at 25°C insoluble N increased from 1.2 mg. N/g. fresh weight to 1.5 mg. — a 25 % increase. As regards the other tissues however a higher level of synthesis has been attained under our conditions than that reported by other workers even in the presence of auxin.

This high level of synthesis is almost certainly related to the presence in the tap water of free nitrate at a concentration of 0.1 ppm. During the experimental period the total N content of beet disks increases from 1 mg./g. to 2 mg./g. (MacDonald *et al.*, 1960) and it has been shown here that in beet disks maintained in distilled water at 25°C net protein synthesis reaches a maximum after only 4 days and this value while still representing a net increase of 100 % is less than that attained during the same period by disks in tap water.

The increase in organic acids in red beet disks (Table 4) is mainly accounted for by accumulation of oxalic acid. This acid has been found to increase in plants which have been supplied with nitrate as nitrogen source (Ruhland and Wetzel 1926) and the accumulation noted here may therefore be due to utilization by the disks of the nitrate absorbed from the tap water. The amino acid content of red beet disks as estimated by decarboxylation is low (Figure 3) and accounts for not more than 20 % of the soluble N. Chromatographic analyses confirmed that no free amino acids remained in the disks after 4 days and it is possible that amides comprise a larger fraction of the soluble N.

Protein synthesis and growth

A comparison of Figures 1 and 2 shows a general correspondence between protein synthesis and fresh weight increase, the beets showing the greatest and swede the smallest changes. Furthermore it is of particular interest that increases in fresh weight of beet disks maintained in both tap and distilled

water show parallel changes to the protein increases. This correspondence is also shown in the differences between tap and distilled water. In distilled water the fresh weight and protein reach a maximum by the 4th day lower than the values recorded in disks in tap water on the same day and in tap water the increase in fresh weight and in protein continues for several days after this. This parallelism between protein synthesis and growth has been shown to occur in the root tips of peas (Brown and Broadbent 1950) and in enlarging cells of the hypanthium of *Oenothera* (Blank and Frey-Wyssling 1944). Thimann and Loos (1957) used auxins to study the relationship between protein synthesis and water uptake in disks of potato and artichoke and concluded that the relationship if any was an indirect one. Their control experiments however demonstrate the effect noted here that the tissue showing the most protein synthesis (potato) has comparatively the greater water uptake. The results presented here indicate that growth in disks is in fact linked to protein synthesis and is limited by the level of soluble N available for synthesis.

Synthesis of other cellular constituents

The data presented in Table 1 indicate that even a 3-fold increase in the protein content of the disks could only account for a fraction (30 % would be a maximum value) of the increase in the dry weight of boiled disks. Obviously therefore material other than protein is being synthesised. Part of the increase in water-insoluble material will be accounted for by the synthesis of TCA-insoluble phosphorus compounds (Table 2). This increase probably represents among other things a synthesis of nucleic acids, phospholipids and other phosphoproteins. In addition an increase in the content of pectic substances in the disks has already been reported (Knight *et al.*, in press). This is in keeping with the finding of Carlier and Buffel (1955) that over a period of several days water uptake by potato tuber tissue is accompanied by synthesis of cellulose and polyuronides. Two of the conditions necessary for the formation of the anhydrogalacturonic acid residues of pectic chains — a source of energy and a supply of glucose units (Seegmiller, Jang and Mann 1956) — can obviously be satisfied in increasing abundance in the disks for almost a week and it is clear from the results already reported that synthesis of cell wall components is achieved.

Conclusion

This investigation taken in conjunction with earlier studies furnishes the materials for drawing an overall picture of the metabolic changes induced in storage tissue by slicing. The first measurable change is an immediate and

progressive increase in the respiration rate (MacDonald and DeKock 1958). This increased respiratory capacity is apparently obligatorily linked to phosphorylation (Laties 1957, Loughman 1957) and the energy thus made available is utilised in endergonic reactions such as salt accumulation (MacDonald *et al.* 1960) and synthesis of cellular constituents (Knight *et al.*, in press). The extent of the growth phenomena induced as reflected by increase in fresh weight together with the nature of the cellular compounds synthesised suggest that growth by cell division supplemented by cell enlargement or *vice versa* occurs. The several features associated with the enlargement and division of meristematic cells — increase in protoplasmic protein, cell wall material, solute and water content — are all observable. Water absorption could be due in part to an increase in cellular osmotic pressure resulting from the hydrolysis of sucrose to glucose and fructose in beet and carrot (MacDonald and DeKock 1958). However uptake continues after complete hydrolysis has occurred and must therefore be mediated by an active mechanism or result from a reduction in wall pressure due to production of more cell wall material.

The precise nature of the stimulus which triggers off the sequence of metabolic transformations in disks remains unknown. Aerobic metabolism is certainly all-important but the theory that the metabolic changes are a consequence of improved oxygen access has been shown to be untenable (Laties 1957). Whatever the cause of the changes occurring in sliced tissue, the changes themselves are clearly of a fundamental nature and are probably most accurately characterised in terms of renewed growth. It would seem too that the rapid development of a capacity for salt uptake which is so unique a feature of disk metabolism is in fact as Steward predicted causally related to protein synthesis not only in potato but also in beet and other storage tissues. The protein synthesized may of course be present and operative in more than one form *e.g.* as protoplasm or in association with carriers or enzymes but the capacity for rapid uptake would seem to be a property of cells which have undergone rejuvenation rather than cells which are either dormant or non-growing.

Summary

Levels trichloroacetic acid insoluble compounds of nitrogen and phosphorus have been followed in disks of sugar beet, red beet, carrot and swede maintained in running tap water, and have been shown to increase in varying degrees in all tissues. Control experiments with disks in distilled water showed that protein synthesis (TCA-insoluble N) is promoted by the pre-

sence of free nitrate (0.1 ppm) in the tap water. The synthesis of insoluble N and P compounds is less than the increase in total water insoluble compounds indicating the synthesis of cell wall materials. Fresh weight increases of up to 70 % are shown to be co-extensive with the synthesis of protein. It is concluded that the metabolic changes occurring in the disks are most accurately characterised in terms of renewed growth. The relationship between growth and metabolism in disks is briefly discussed.

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Studies on an Unknown Metabolic Product of 3-Amino-1,2,4-triazole

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Introduction

3-Amino-1,2,4-triazole (ATA) has received considerable attention by researchers because of its effectiveness as a phytocide and defoliant (7) and its pronounced effect upon chlorophyll synthesis (9). If the mechanism of action of ATA could be determined, the effectiveness of the compound as a phytocide might be improved and valuable information concerning chlorophyll synthesis may be obtained.

Several workers have investigated the fate of ATA in plants (1, 5, 10, 11). Their work indicates that metabolic products of ATA may play an important role in the toxic action of the compound. One metabolic transformation product in particular occurs in considerable quantities in a variety of species (5, 10). The derived compound has been shown to contain not only the number 5 carbon atom of ATA but also carbon atom(s) from glycine or serine (3, 4). This unknown which we will designate compound "1" was biosynthesized, isolated and subjected to a number of tests so that some of the chemical characteristics could be determined.

Materials and Methods

Production of the Unknown. — To produce non-radioactive compound "1", approximately 1,000 bean plants (*Phaseolus vulgaris* var. Black Valentine) were grown in the greenhouse as previously described (5). When the plants were 7 days

old from seed, they were treated with an aqueous solution containing 0.1 M ATA, 0.01 M glycine, and 0.1 % Tween 80. The solution was applied to one primary leaf with a small paint brush. A treatment such as this was generally sufficient to produce nearly complete chlorosis of the terminals and first trifoliates, but still not kill the plants.

Chlorotic tips and trifoliates were harvested 5 days after start of the treatment, i.e., 12 days after planting. Approximately 35 grams, fresh weight, of tissue were obtained. The tissue was boiled for 2 minutes in 300 ml. of 80 % ethanol, filtered through 2 layers of cheesecloth and then ground with water in a Ten-Brock homogenizer. The homogenate and ethanol extract were combined and filtered 3 times through Whatman no. 1 paper, the residue being washed each time with 25 ml. of boiling distilled water.

The combined extracts were fed onto a 0.9×10 cm. column of Dowex 50×8 cation exchange resin in the hydrogen form and washed with successive 100 ml. aliquots of distilled water, benzene, 80 % ethanol, and distilled water. Elution was carried out with a straight line gradient from 0 to 0.1 N HCl in 1000 ml. of solution followed by 0.1 N to 1.0 N HCl in a second 1000 ml. of solution. A constant pressure of 1.0 psi was maintained on the column. Fractions of from 4.5 to 5.0 ml. were obtained at two-minute intervals until a total of 420 fractions had been collected. Compound "1" came off in tubes 397 to 417. To reduce the volume of the eluate containing compound "1", it was passed through a 0.5×0.9 cm. column of Dowex 50×8 and eluted with 1.0 N NH_4OH . All of compound "1" came off in the first 5 ml. of NH_4OH eluate.

The preparation of compound "1" was further purified by paper chromatography on Whatman No. 3MM paper using the strip method. Separation was effected first with 71 % phenol, then butanol-propionic acid-water, and finally 80 % ethanol. After each chromatographic run, the strip containing "1" was cut out and eluted. Both the chromatograms and eluates were washed with diethyl ether after the phenol run.

Chromatography and electrophoresis indicated that the final preparation of compound "1" contained traces of glutamine and possibly glycine. The purity was estimated to be 95 to 97 %.

Some radioactive compound "1" was also produced. This was done using unlabeled ATA and glycine- 1-C^{14} — designated "1-A"; unlabeled ATA and glycine- 2-C^{14} — designated "1-B"; ATA- 5-C^{14} and glycine- 1-C^{14} — designated "1-C"; ATA- 5-C^{14} and glycine- 2-C^{14} — designated "1-D"; ATA- 5-C^{14} and glycine- U-C^{14} — designated "1-E"; ATA- 5-C^{14} and unlabeled glycine — designated "1-F".

Detached tips and trifoliates of 14-day-old bean plants were placed in glass vials containing 1 microcurie of labeled glycine in 0.1 M ATA, 1 microcurie of labeled ATA in 0.1 M glycine or 0.5 microcuries of labeled glycine and 0.5 microcuries of labeled ATA. Three tips were used for all preparations except "1-F" for which 43 tips were used. Feedings were carried out for 24 hours in constant illumination (600 fc.). Extraction and purification procedures were the same as for the unlabeled compound "1" except the gradient elution step was omitted. The preparations contained traces of several contaminants but neither glycine nor ATA could be detected in any of the preparations.

Hydrolysis of compound "1". — Aliquots (from 200 to 500 cps.) of each preparation of radioactive compound "1" were placed in glass tubes containing 1.0 N HCl or 1.0 N NH_4OH . The tubes were sealed and placed in an oven at 90°C . for

1 hour. Other aliquots were placed in tubes of 6.0 *N* HCl, sealed and placed in a steam autoclave for 16 hours. ATA-5-C¹⁴ and glycine-U-C¹⁴ were also hydrolysed. Chromatography and autoradiography were carried out as previously described (5).

Volatility of the unknown. — A 0.025 ml. sample of "1-F" was placed in each of 9 nickel planchets. The planchets were divided into 3 groups of 3; one group was held at 90°C., another at 20°C., and the third group at room temperature. The planchets were counted periodically for 6 weeks.

Electrophoresis. — The behaviour of compound "1" to electrophoresis at various pH levels was studied using a Spinco "Durrum" type apparatus. A potential of 1000 v was maintained for 2 hours. Standard Harleco buffer salts were used at a concentration of 0.5 %.

Color reaction. — Compound "1" was tested for its reaction to ninhydrin, Ehrlich's (*p*-dimethylaminobenzaldehyde in ethanolic-HCl), *p*-anisidine (2), "H-acid" (10), HCl-phenol (1), and nitroprusside-ferrocyanide (12).

Metabolism and phytotoxicity of "1". — Radioactive compound "1" was fed to the excised tips of 14-day-old bean plants by the usual method (5).

To determine the phytotoxicity of compound "1", a number of bean plants were treated with a solution of unlabeled "1" on one primary leaf while other plants were treated by placing a droplet of solution directly upon the tips. For comparison, a number of plants were treated with 0.1 *M* ATA and others with a solution of 10 common amino acids (each at 1 mg/ml.) in the same ways that the "1" was applied.

Results

Compound "1" was not hydrolyzed by 1.0 *N* HCl nor 1.0 *N* NH₄OH after one hour at 90°C. Partial hydrolysis was accomplished with 6.0 *N* HCl after 16 hours in an autoclave (Table 1) (Figure 1 a, b, c, d). The unknown appeared to be quite stable.

Radioactive ATA was regenerated from all preparations of compound "1" produced from ATA-5-C¹⁴ originally (Figure 1 b and 1 d). The only other hydrolytic products which appeared in appreciable quantities were derivatives of ATA which we had previously discovered in plants (5) (Figure 2). No compound which might represent the glycine moiety of compound "1" was apparent on the chromatograms.

Compound "1" did not appear to be volatile (Table 2). After 6 weeks at 90°C. there was no appreciable decrease in the radioactivity of a sample of "1-F". The samples stored at room temperature showed a slow but steady decrease (Table 2). These samples were exposed to light while the samples at 90°C. and -20°C. were stored in darkness. Exposure to light may have caused some breakdown and loss of "1", however, the noted decrease in activity was not statistically significant.

Table 3 shows the pattern of movement of compound "1" during electrophoresis at different pH levels. The behavior of compound "1" was typical

Table 1. The results of 6.0 N HCl hydrolysis of radioactive Compound "1"¹ formed by bean plants from various substrates. (Compound "1-A" formed from glycine-1-C¹⁴ and ATA (unlabeled); "1-B" from glycine-2-C¹⁴ and ATA (unlabeled); "1-C" from glycine-1-C¹⁴ and ATA-5-C¹⁴; "1-D" from glycine-2-C¹⁴ and ATA-5-C¹⁴; "1-E" from glycine-U-C¹⁴ and ATA-5-C¹⁴; "1-F" from glycine (unlabeled) and ATA-5-C¹⁴). The radioactivity in the various compounds is expressed as a percentage of the total on the chromatograms.

Compound ² Derived	Substrate undergoing hydrolysis					
	"1-A"	"1-B"	"1-C"	"1-D"	"1-E"	"1-F"
"1"	29.0	71.4	29.9	53.0	40.7	76.4
"2" & "8"	25.5	3.9	13.1	5.9	9.1	—
"9"	9.9	5.0	16.5	7.2	8.4	—
"10"	27.3	14.1	25.0	17.9	25.2	3.3
ATA	—	—	6.7	11.3	9.5	20.3
Other ³	8.2	4.9	8.8	4.8	7.0	trace

¹ All preparations of radioactive Compound "1" were at least 90 % pure on the basis of total radioactivity as determined by two-dimensional paper chromatography, filming, and counting.

² Numbers refer to compounds in Figure 2.

³ "Other" refers to compounds which were contaminants in the original preparation or did not appear to be one of the compounds in Figure 2.

for that of "zwitterion", changing its charge around pH 7.0. Note that mobility was much greater in the anionic than the cationic form.

The reactions of compound "1" to several color producing reagents are shown in Table 4. In general, "1" gave the same reactions to indicator sprays as ATA. The reaction of "1" with "Ehrlich's" was much stronger than the reaction of ATA and "1" gave a positive reaction with ninhydrin while ATA did not. The reaction of "1" and ATA with "H-acid" was not the same after chromatography in phenol as before. The "H-acid" reaction involves diazotizing the ATA ring and coupling it with "H-acid" (8-amino-1-naphthol-3,6-disulfuric acid) to form a red color (10). However, phenol will also react with ATA following diazotizing (1). Evidently, enough phenol remained on the air-dried papers to react with "1" and ATA since both compounds gave a yellow color after chromatography in phenol but a bright red color before chromatography.

Compound "1" appeared to be metabolically inert. Bean plants were unable to metabolize the labeled compound and showed no damage after the unlabeled "1" was placed upon leaves or tips. Plants treated in a similar manner with ATA showed typical toxic effects while the amino acid treatments were non-injurious.

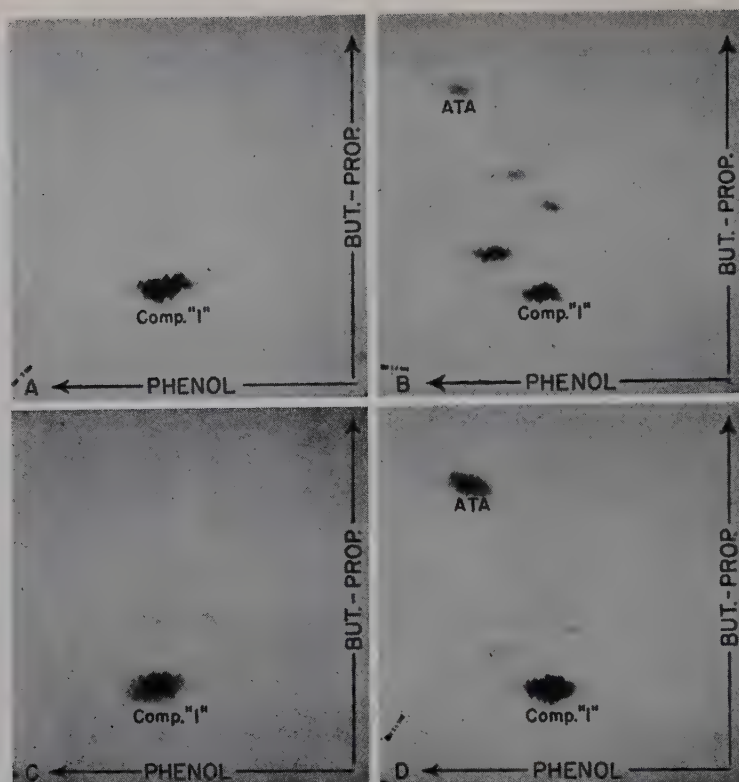


Figure 1. Autoradiograms of two dimensional chromatograms showing (A) "1-E" before hydrolysis; (B) Compounds present after hydrolysis of "1-E"; (C) "1-F" before hydrolysis; (D) Compounds present after hydrolysis of "1-F".

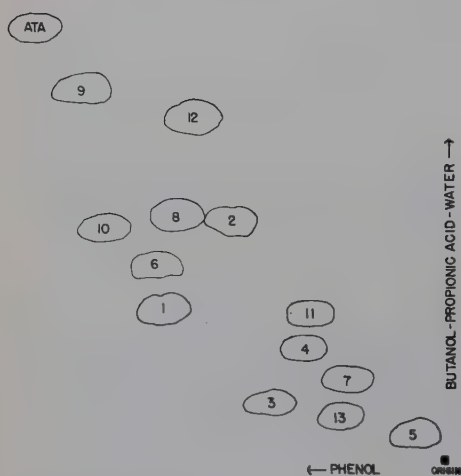


Figure 2. Chromatographic map of derivatives of ATA-5-C¹⁴ which occur in plants. Numbers have been arbitrarily assigned to the unidentified compounds (5).

Table 2. *The results of studies of the volatility of compound "1-F". Each figure represents the average of duplicate countings per second of each of three samples.*

Conditions	Time in days after plating samples					
	0	2	7	14	28	42
Room Temperature (Light)	26.9	25.5	24.9	23.9	23.8	23.6
—20°C (dark)	26.5	26.7	26.4	26.2	25.5	25.8
90°C (dark)	27.7	27.7	28.0	27.6	27.1	27.2

Table 3. *Mobility of compound "1" during paper electrophoresis at different pH values.*

Movement toward the cathode is indicated by a (—) and toward the anode as (+).

pH	Distance moved (mm) ¹
3.0	— 12.4
4.2	— 13.0
5.4	— 5.3
6.0	— 5.6
6.6	— 6.3
7.2	+ 5.7
7.8	+ 16.7
8.4	+ 35.0
9.0	+ 47.3

¹ Distance compound moved in 2 hours with initial potential of 1000 v; figures represent averages of 3 determinations.

Table 4. *Color reactions of compound "1" and ATA.*

Color reagent ¹	Compound "1"	ATA
Ninhydrin	Blue-green	No visible reaction
Ehrlich's	Yellow	Faint yellow
<i>p</i> -Anisidine	No visible reaction	No visible reaction
Phenol-HCl	Yellow	Yellow
"H-acid"	Yellow or red ²	Yellow or red
Nitroprusside-ferrocyanide	Green	Green

¹ See text for details and references to color reagents.

² The "H-acid" reagent produced a yellow color with "1" on chromatograms but a red color on paper before chromatography. A possible explanation is given in text.

Discussion

Studies of the properties of compound "1" indicate that it is a very stable compound. After 16 hours in 6.0 *N* HCl in an autoclave at 15 lbs. pressure "1" was only partially hydrolyzed (Table 1). These findings make it unlikely that "1" is a complex with a protein or a simple sugar. Such was postulated

by Rogers (11) and Gentile and Frederick (6) for ATA derivatives which they observed.

The behavior of compound "1" during electrophoresis indicates that it possesses both acid and basic groups with an isoelectric point around pH 7.0 (Table 2). These findings together with the color reactions of "1" with various sprays (Table 4) leave little doubt that compound "1" is the same material as compound "X" described by Racusen (10), but it might not be the same as Massini's ATX (8) inasmuch as the color developed upon spraying with nitroprusside-ferrocyanide is not the same.

Compound "1" appears to contain intact ATA because "1" gives many of the same color reactions as the phytocide (Table 4) and yields ATA upon acid hydrolysis (Table 1). Other hydrolytic products of "1" were compounds "2", "8", "9", and "10", which, like "1", must contain the number 5 carbon atom of ATA plus a glycine fragment since they were produced in quantities from ATA-5-C¹⁴ alone (5) and appeared as hydrolytic products of "1-A" and "1-B" (Table 1) which contained radioactivity from glycine only. No non-ATA-containing hydrolytic products of "1" were detected. The data indicate that "1" contains several moles of ATA per mole of "1" and/or hydrolysis occurs at several points in the molecule. In either case, any hydrolytic product of "1" representing only the glycine contribution to the molecule is lost during hydrolysis and chromatography. This would be true if the glycine moiety were acetate or formate, for example.

The findings of the present investigation indicate that compound "1" is probably a "detoxification" product and not the active form of ATA. Compound "1" appeared to be metabolically inert and produced no toxic effects on beans when applied externally. These conclusions concur with those of Racusen (10) who also postulated that compound "1" was a detoxification product. However, as suggested earlier (4), compound "1" may be a direct product of the toxic reaction of ATA and a complete description of the chemical structure of "1" may give an insight to the phytotoxic mechanism of the phytocide.

Summary

1. An unknown metabolic product of 3-amino-1,2,4-triazole (ATA) was biosynthesized from ATA-5-C¹⁴, glycine-1-C¹⁴, and glycine-2-C¹⁴ alone and in combinations. The various preparation of the unknown, designated compound "1", were subjected to several chemical and physical tests.

2. Compound "1" was found to be a "zwitterion". The unknown gave all of the color reactions to which ATA reacted positively. In addition it was ninhydrin positive.

3. Prolonged hydrolysis of compound "1" resulted in only partial breakdown. ATA was recovered under these conditions but the other hydrolytic products have not been identified.

4. Compound "1" was not metabolically altered by beans in 24 hours and appeared to be non-phytotoxic.

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The Control of Chlorophyll Accumulation in Leaves of Marquis Wheat by Temperature and Light Intensity

II. Chlorophyll Contents Relative to Leaf Area and Thickness

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Introduction

In the first part of this paper (3) it was seen that the amount of chlorophyll formed per leaf was dependant on the temperature and light conditions under which the plant had been grown. The concentration of chlorophyll expressed as a percentage of the leaf fresh weight, was related to light intensity and temperature in a similar manner as the chlorophyll content per leaf, except at 30°C, where the concentration was higher than at 25°C. This was attributed to the large decrease in leaf fresh weight at 30°C. The effect of light intensity on chlorophyll concentration at 30°C was similar to that measured on a per-leaf basis. The Q_{10} for chlorophyll formation in leaves of plants grown continuously in light was the same as that for etiolated seedlings as reported by Lubimenko and Hubbenet (9), and can be attributed to the known effects of temperature on protochlorophyll formation and transformation, as studied by Virgin (13), Smith (10) and others.

The effect of light intensity on the plants grown continuously in light cannot be simply related to the reported effects of light on chlorophyll formation in etiolated seedlings. Unlike temperature, where energy is received by the plant in proportion to the volume of material present, light energy is strongly directional, so that the total amount received by any one leaf will depend on its shape and size.

In the dark-grown seedling protochlorophyll is converted by light into chlorophyll *a*. This reaction is very rapid, and is predominantly photochemical: considerable conversion takes place even at -70°C (11). The threshold light intensity for the conversion in etiolated *Phaseolus* plants was determined as above 0.005 lux (0.00047 ftc.) (5), and the rate of transformation progressively increases with light intensity from 30 to 240 ftc. in etiolated corn (7). At high light intensities the transformation may be inhibited (13). The action spectrum for the conversion of protochlorophyll in etiolated leaves corresponds with the absorption of protochlorophyll itself (6).

After the initial protochlorophyll conversion, further accumulation of chlorophyll is affected by light in several ways. The rate of formation and of transformation of protochlorophyll, through which further chlorophyll *a* is formed, is itself affected by light intensity (13). An indirect effect on protochlorophyll formation through the control of photosynthesis by light has also been suggested (12). As chlorophyll accumulates, the light energy available for chlorophyll formation in deeper tissues of the leaf will be reduced in intensity and altered in spectral characteristics towards the green, because of the absorption of the chloroplast pigments. It is known that green light is not as effective as red or blue in the conversion of protochlorophyll to chlorophyll (2).

Experiments to determine the relative importance of these several actions of light on chlorophyll formation were carried out in dark-grown seedlings. The results given in part one of this paper (3) are examined in regard to the effects of light intensity on leaf area and thickness, and its relationship to chlorophyll concentration.

The Relation between Leaf Area and Thickness and the Maximal Chlorophyll Content of Plants Grown under Continuous Illumination

Method

The plant material obtained in the previously described experiments (3), was placed in a photo-electric planimeter (1) for the determination of leaf area. Numbers of replicates and the analysis of the results were the same as those previously described for chlorophyll determinations. Leaf thickness was measured by a micrometer adjusted so as not to crush the leaves. Each leaf was split down one side of the mid-rib, and the thickness of the lamina minus mid-rib was measured one centimetre from the base and tip of the leaf, and in the centre of the leaf. At any one harvest date three pots of four plants were used, and the first three leaves on the main shoot were measured separately. Mean values for any one leaf position on any one sampling occasion thus represent 36 micrometer readings.

Results

In the results given in part one of this paper (3) it was shown that the time of beginning chlorophyll formation and the rate of chlorophyll formation differed in the 1st, 2nd and 3rd leaves. Because comparison of chlorophyll in leaves of the same age would have resulted in comparison of leaves in different stages of morphological and physiological development, the effect of the environment on chlorophyll formation was expressed by comparing leaves at the same stage of a particular physiological development, namely the stage of maximal chlorophyll content. For any one leaf this maximum chlorophyll content coincided with the time of maximal leaf area, fresh and dry weight.

There was no allometric relationship between chlorophyll content and leaf area or weight because chlorophyll formation did not take place to any extent until the leaf had become exposed to light by growth through the sheaths of preceding older leaves. The rate of chlorophyll formation must therefore have been greater than that of increase in area or weight, because the maxima occurred at the same time. This is shown by the increasing value with time of chlorophyll expressed as a percentage of fresh weight (Table 1). Chlorophyll per unit leaf area showed similar results.

The maximum chlorophyll content expressed as mg. per dm² of leaf area (Table 2) showed similar relationships to light and temperature as those of chlorophyll expressed as percentage fresh weight (3, Table 4). The reduction in the leaf area at 30°C compared with that at 25°C was even more marked than the reduction in fresh weight, so that the values of chlorophyll content per unit area were on the average twice those of the corresponding values at 25°C.

Table 1. *Chlorophyll as a percentage of leaf fresh weight, and its relationship with time.* Figures are for the chlorophyll content of the second leaf, grown at 20°C. Each figure is the mean of 12 leaves.

Age of plant days	Light intensity				
	200	500	1,000	1,750	2,500
9	0	0	0.15	0.13	0.15
12	0	0	0.13	0.20	0.22
14	0.08	0.13	0.25	0.24	0.27
16	—	—	0.26	0.28	0.28
21	0.08	0.15	0.26	0.30	0.31
28	0.09	0.14			

Time of maximal chlorophyll content per leaf in italics.

Table 2. *The mean values of the maximal chlorophyll contents of the first three leaves expressed as mg. per dm². leaf area, as affected by temperature and light intensity.*
S.E. of means = 0.36

Light Intensity ftc.	Temperature °C				
	10	15	20	25	30
200	2.3 ¹	2.3	1.7	1.7	5.7
500	3.3	2.8	2.3	2.3	5.6
1,000	4.2	4.5	4.5	4.0	7.2
1,750	4.4	4.9	4.9	4.7	8.8
2,500	2.6	4.5	5.7	5.4	10.6

¹ Mean of 36 leaves.

The differences in chlorophyll content between the first three leaves noted in Part 1 can largely be attributed to the differences in their areas. The values of chlorophyll per unit area are given in Table 3. The second leaf in general had a lower content than the first or third. A comparison of the two measures of chlorophyll content, shows that when expressed as a percentage of the content of leaf three, the values for chlorophyll content on an area basis were much more uniform than the absolute ones (Table 3).

Table 3. *Variation of chlorophyll content with leaf position.*
Chlorophyll content as mg. per dm². Each figure is a mean of 15 leaves.

Leaf	Temperature °C				
	10	15	20	25	30
1	2.9	2.6	3.1	3.0	5.5
2	2.0	2.7	2.6	2.5	4.7
3	2.7	2.8	2.7	2.4	4.2

S.E. \pm 0.19

Leaf	Light intensity ftc.				
	200	500	1,000	1,750	2,500
1	2.3	2.8	3.8	4.1	4.1
2	1.5	2.2	3.2	3.7	3.8
3	2.4	2.2	3.6	3.9	4.3

S.E. \pm 0.19

Chlorophyll content as per cent of leaf 3.

Leaf	mp. per leaf	mg. per dm ²
1	43	114
2	59	97
3	100	100

Table 4. *The effect of temperature and light intensity on leaf thickness at the time of maximal chlorophyll content.* Thickness in mm., lamina measured in three places for each leaf, each figure represents measurements from 12 leaves. Figures are means of the first three leaves.

Light intensity ftc.	Temperature °C				
	10	15	20	25	30
200	0.16	0.15	0.14	0.13	0.15
500	0.18	0.17	0.17	0.14	0.16
1,000	0.23	0.23	0.20	0.18	0.17
1,750	0.24	0.25	0.21	0.18	0.19
2,500	0.25	0.25	0.23	0.20	0.19

S.E. \pm 0.002

The values of leaf thickness at the time of the maximal chlorophyll content are given in Table 4. A strong correlation was found between chlorophyll contents per unit area and leaf thickness, as shown by the correlation coefficients given in Table 5. The regression of chlorophyll content on leaf thickness was linear at each temperature (omitting the 2,500 ftc. values at 10 and 15°C, where the low temperature probably allowed the dominance of light destruction of pigment) (Figure 1). The effect of temperature on chloro-

Table 5.

A. *Correlation coefficient of chlorophyll content per unit leaf area with leaf thickness produced by different light intensities.*

Temperature °C	Correlation coefficient	Significance
10	0.470 (0.975) ¹	> 0.1 (0.01)
15	0.978 (0.989) ¹	< 0.01 (0.01)
20	0.983	< 0.01
25	0.996	< 0.001
30	0.907	< 0.05

¹ Omitting 2,500 ftc. values.

B. *Correlation coefficient of chlorophyll content per unit leaf area with leaf thickness produced at different temperatures.*

Light intensity ftc.	Correlation coefficient	Significance
200	0.210	> 0.05
500	— 0.266	> 0.05
1,000	— 0.477	> 0.05
1,750	— 0.473	> 0.05
2,500	— 0.816	> 0.05

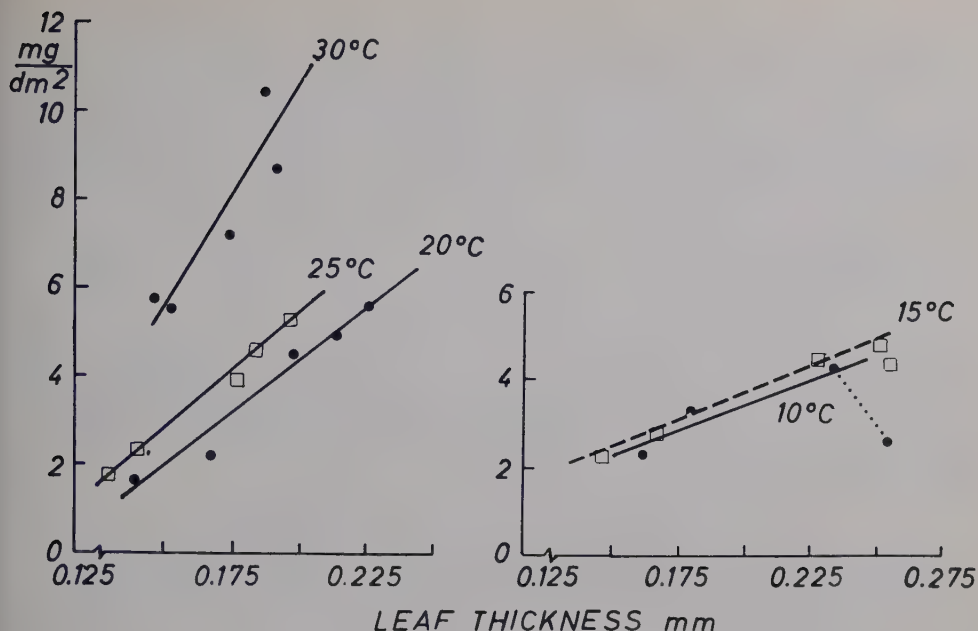


Figure 1. The regression of chlorophyll content expressed as mg. per dm². of leaf, on leaf thickness, at different temperatures. Values are the mean maximal chlorophyll contents of the first three leaves. Regression coefficients of chlorophyll content on leaf thickness

10°C	0.76
15°	0.84
20°	1.66
25°	1.79
30°	3.27

phyll content cannot be attributed to parallel changes in leaf thickness as the correlation coefficients were not significant (Table 5 B).

To determine whether light intensity had any effect on chlorophyll concentration in addition to its indirect effect through leaf thickness, a multiple regression analysis was carried out.

Chlorophyll content per unit leaf area was used as the dependent variable, using all values, and (i) coded temperature (denoted by t), (ii) (coded temperature)², (iii) coded leaf thickness (denoted by T), (iv) (coded leaf thickness)², (v) (coded temperature) \times (coded leaf thickness), and (vi) coded light intensity, as independent variables. Temperatures were coded using 0 for 10°C, 1 for 15°C, etc. up to 4 for 30°C. Leaf thickness was coded by multiplication by 30. Light intensity was coded by dividing by 50. These codings were used to simplify the arithmetic procedures.

The multiple regression was highly significant, the multiple correlation coefficient being 0.95. It was found that the partial regression on light intensity was not significant, and was indeed smaller than its standard error. Light therefore has no effect on chlorophyll content other than through its effect on leaf thickness, in these experiments. It was further found that virtually all of the sum of squares for the regression term was contributed by only three of the above listed six independent variables, leading to the following equation.

$$\bar{y} = 3.55 - 5.66 t + 0.65 t^2 + 0.79 tT \quad (\text{S.D.} = 0.70).$$

This confirms the results seen in Figure 1, that temperature has a large direct effect on chlorophyll content, and that there is also linear regression on leaf thickness, which also contributes to the effect of temperature on chlorophyll content.

The Effect of Light Intensity on the Accumulation of Chlorophyll in Dark-grown Seedlings of Marquis Wheat

Methods

Seeds were planted in vermiculite-gravel medium, soaked with Hoagland's No. 1 nutrient solution, and placed at 23°C in the dark for seven days. They were then watered again with nutrient and placed under a range of light intensities in a growth cabinet. Illumination was provided by standard cool white fluorescent lamps and supplemented by incandescent light from 60 watt bulbs in the ratio 1,000 ftc. fluorescent to 30 ftc. incandescent. The light intensity was varied by altering the distance of the seedlings from the light source. In the first experiment the temperature was maintained constant at 23°C. This temperature was chosen as being near the maximum for greening (3) and because small changes in temperature between plants exposed to different light intensities would have a minimal effect, as the Q_{10} for chlorophyll formation is about 1 from 20–30°C (3, Table 2).

The initial conversion of protochlorophyll formed in the dark was allowed to proceed in dim light (100 ftc.) for 10 minutes before transfer of the plants to a range of light intensities for further chlorophyll accumulation. At the time of exposure to light the 1st leaf was fully emerged and the 2nd leaf just visible. The plants were exposed horizontally to light intensities ranging from 2 ftc. to 117 ftc. A background of uniform reflectance was provided by laying the pots containing the seedlings horizontally upon paper towelling. After 17 hours, two samples of 10 leaves were taken from each intensity and the chlorophylls extracted by acetone end estimated in a Beckmann spectrophotometer, as previously described in Part I.

In the second experiment the plants were exposed horizontally to a series of light intensities ranging from 200 to 2,200 ftc. The temperature was maintained constant at 26°C for the plants at 1,400 ftc. and because of the energy produced from the light panels, varied from 28°C for the 2,200 ftc. plants to 23°C for the 200 ftc. series.

In a third experiment plants were grown in the dark as before, but at a temperature of 20°C instead of 23°C. After seven days the plants were not as advanced in growth as in the first two experiments and the 2nd leaf was not visible. The plants were placed under light intensities ranging from 2 ftc. to 1,000 ftc. for 18 hours at a temperature of 22–23°C, except at 1,000 ftc., where the temperature was 26°C.

Results

Table 6 A gives the amount of chlorophyll *a* plus *b* formed per leaf, on a fresh and dry weight basis. The dry weights were calculated by assuming

Table 6. *The effect of different intensities of white light on the formation of chlorophyll in the first leaf of dark-grown seedlings of Marquis wheat.*

A. *First experiment* Exposed to light for 17 hours at 23°C.

Light intensity ftc.	Chlorophyll a + b mg.		
	per 10 leaves	per 10 g. fresh weight	per 10 g. dry weight
2	0.14	0.29	3.4
24	0.16	0.34	4.1
52	0.19	0.40	4.4
120	0.23	0.50	5.5
170	0.24	0.50	5.5

B. *Second experiment* Exposed to light for 19 hours at 26°C.

Light intensity ftc.	Chlorophyll a + b mg.		
	per 10 leaves	per 10 g. fresh weight	per 10 g. dry weight
200	0.21	0.45	4.5
975	0.24	0.52	4.9
1,400	0.25	0.54	5.2
1,700	0.22	0.49	4.4
2,200	0.23	0.49	4.4

Each number represents the mean of two groups of 10 plants.

C. *Third experiment* Exposed to light for 18 hours at 23°C.

Light intensity ftc.	Chlorophyll a + b mg.	
	per 10 leaves	per 10 g. fresh weight
2	0.25 S.E. \pm 0.010	0.57
20	0.23	0.63
230	0.31	0.82
1,000	0.34	0.98

Each number represents the mean of 4 groups of 10 plants.

an equal relationship of dry to wet weights as found in parallel samples. The chlorophyll concentration was markedly less on all bases of estimation below 120 ftc. From the second experiment (Table 6 B), there was slightly lower chlorophyll content at the higher light intensities: the maximal content was reached under the 1,400 ftc. series. This was evident when chlorophyll was expressed either as mg. per leaf or on a wet or dry weight basis.

The results of the third experiment (Table 6 C) cannot be directly compared with those of the first two experiments because of the smaller initial size of the dark-grown seedlings. There was again an increase in chlorophyll from the 2 ftc. to the 1,000 ftc. variants on all bases of estimation, although above 230 ftc. this increase was small.

The Influence of the Age of the Dark-grown Leaf on the amount of Chlorophyll Formed on Transfer to Light

From the previous results it was seen that there was markedly less chlorophyll accumulated below an intensity of 230 ftc. The declining rate of chlorophyll formation near the maximal content (3, Figure 1) in plants grown continuously in the light might therefore be caused by an increasing internal absorption of light by the accumulating pigments, so that the light intensity within the leaf was below the optimum for the protochlorophyll-chlorophyll conversion. An alternative explanation of the declining rate of chlorophyll formation is that the metabolic activities of the leaf change with age in the direction of increasing dominance of chlorophyll breakdown over chlorophyll synthesis. The following experiment was undertaken to test this latter alternative.

Plants were grown in the dark at 21°C as previously described and after 4, 5, 6, 7, and 8 days were transferred for a period of 24 hours to light of 1,000 ftc. intensity at a temperature of 23°C. Plants were also grown under continuous illumination from germination.

Results

The chlorophyll content expressed as a percentage of fresh weight is given in Table 7. The daily increase in chlorophyll content ran parallel in both the dark-transferred and continuously illuminated plants, and declined in both series after 7 days growth. At this time the first leaf was fully expanded in both series and the second leaf had emerged to a length of about 4 cm.

Table 7. *The influence of the age of the etiolated first leaf on the amount of chlorophyll formed on transfer to light.*

A.

Age at time of transfer to light, days	Total chlorophyll	
	mg. per 10 leaves	mg. per 10 g. fresh weight
4	0.48 \pm .021	1.1 \pm .047
5	0.53 \pm .021	1.0 \pm .047
6	0.53 \pm .021	1.1 \pm .047
7	0.44 \pm .021	0.9 \pm .047
8	0.29 \pm .021	0.6 \pm .047
Age of plants grown continuously in light, days		
4	0.4 \pm .048	1.4 \pm .034
5	1.0 \pm .048	1.5 \pm .034
6	1.5 \pm .048	1.7 \pm .034
7	1.5 \pm .048	1.8 \pm .034
8	1.7 \pm .048	2.0 \pm .034
9	1.6 \pm .048	1.9 \pm .034

B. Plants grown continuously in light.

Interval day	Daily increase in chlorophyll	
	mg. per 10 leaves	mg. per 10 g. fresh weight
4 — 5	0.51	0.04
5 — 6	0.43	0.16
6 — 7	0.04	0.15
7 — 8	0.22	0.17
8 — 9	0.07	0.04

Each figure is the mean of 3 replicate samples of 10 plants.

Discussion

In the experiments on etiolated seedlings, the effects of light intensity on chlorophyll formation can be studied without the complications introduced by the differences in leaf area and thickness that take place when leaves are grown from germination under a range of light intensities, as the structure of the etiolated leaves was largely determined before their transfer to light. Light saturation was reached at about 1,000 ftc., in contrast to the leaves grown continuously in the light, where an increase was found up to 2,500 ftc. There was a strong correlation of chlorophyll content with leaf thickness in these latter plants, with no residual effect of light (Table 5). The linearity

of the relationship shows that even though the light intensity inside the leaf must be reduced by absorption by the chloroplast pigments, and even though the spectrum is also changed in the direction of wavelengths of decreasing effectiveness, light was still saturating for chlorophyll accumulation at the sites available. Under conditions in which leaf thickness increased, the sites of chlorophyll formation (presumably a function of the number of cells and number of potential chloroplasts per cell) also increased proportionally. Only under conditions of low temperature and high light intensity (10°C and to a lesser extent 15°C under a light intensity of 2,500 ftc.) did this relationship break down. It is unlikely that the leaf thickness was controlled by a restriction of photosynthesis determined by the chlorophyll content, as it has been shown (4) that the rate of photosynthesis only begins to be affected when the chlorophyll content is below 4–5 mg. per dm^2 . and then only at low light intensities.

Changes in chlorophyll content induced by temperature, unlike those induced by light intensity, reflect changes in metabolic processes involved in chlorophyll synthesis rather than an effect on leaf thickness. The effects of temperature are therefore similar in both etiolated and continuously light grown plants, as shown by the Q_{10} 's of the reaction. (3, Table 2).

Further evidence that internal absorption of light by the leaf pigments does not limit the effect of light on chlorophyll formation was obtained in the experiments with etiolated seedlings. There was a parallel decrease in the rate of chlorophyll accumulation in both the transferred plants and those grown continuously in light, as the leaf aged (Table 7). This indicates that the phase of declining rate of chlorophyll accumulation in plants grown continuously in the light is related to a change in the balance of chlorophyll synthesis and destruction with time, rather than a reduction of light by internal absorption of light as the leaf approaches its maximal chlorophyll content.

Summary

1. The control of chlorophyll accumulation by light intensity in the first three leaves of Marquis wheat was through parallel changes in leaf thickness.
2. The effect of temperature was primarily on metabolic processes connected with chlorophyll formation, with a similar Q_{10} to that found for the accumulation of chlorophyll in etiolated seedlings as reported by other workers.
3. There was a small additional effect of temperature on chlorophyll content per unit leaf area, through the temperature control of leaf thickness.

4. Differences in chlorophyll content between the first three leaves on the main shoot were related to differences in leaf area and weight.
5. The accumulation of chlorophyll in etiolated leaves increased with light intensity up to 1,000 ftc. The rate of accumulation was related to the age of the etiolated leaf, the decline occurring at the same time as in plants grown continuously in the light.

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Effect of *p*-Chlorophenoxyisobutyric Acid on Rate of Elongation of Root Hairs of *Agrostis alba* L

By

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Introduction

Several hypotheses have been proposed to explain the demonstrated anti-auxin action of *p*-chlorophenoxyisobutyric acid (PCIB). Burström (1950) found that PCIB caused an increase in the rate of elongation of epidermal cells of wheat roots and concluded that its action was opposite to that exerted by indoleacetic acid (IAA), *i.e.*, PCIB slows down the first phase of cell elongation (cell stretching), while accelerating the second (cell wall synthesis). Since the second phase dominates, the overall effect of PCIB is to stimulate root growth. McRae and Bonner (1953), utilizing the Lineweaver-Burk plotting method, undertook a mathematical analysis of the interaction of PCIB and IAA on elongation of oat coleoptile sections. They found PCIB to be an inhibitor of IAA and explained this as a competition for auxin-reactive sites. Fransson (1958) reported that wheat roots contain free IAA which is easily extracted and a bound form from which free IAA is released. Treatment of roots with PCIB resulted in an increase in the concentration of free IAA, thus providing additional evidence that PCIB competes with IAA. Since it is well known that PCIB stimulates root cell elongation, Fransson concluded that the competition between the antiauxin and the natural auxin takes place where auxin exerts its elongation activity and that the free, non-coupled IAA is not directly active in cell elongation.

It is assumed by many workers (*cf.* Åberg 1957) that the response of roots to exogenous auxin is fundamentally different from the response of coleoptiles, stems, and buds. That is, that auxin, even at very low concentrations,

inhibits root elongation. The numerous reports of stimulation of root growth by auxins are dismissed (Åberg 1957) on the grounds that the results were obtained under unusual conditions, *i.e.*, on isolated roots or only after the roots had been exposed to auxin for long periods of time. It has been claimed (Burström 1942, Lundegårdh 1946, Ekdahl 1953) that concentrations of IAA which inhibit root growth have little or no effect on root hairs. Jackson (1960) has recently shown that the dose-response curve for effects of IAA on rate of root hair elongation is similar to that for coleoptiles, buds, and stems, but the optimum concentration (10^{-13} M) is several orders of magnitude lower than that for the above mentioned structures.

Although Burström (1950) found that PCIB prevented *initiation* of root hairs while stimulating root growth, no mention was made of its effects on rate of elongation of root hairs growing at time of treatment. Ekdahl (1957) mentioned, without presenting data, that the maximum length of root hairs was strongly changed; increased in auxin media and decreased in antiauxin media. These results, and the results of Jackson (1960) on the effect of indoleacetic acid on the rate of elongation of root hairs of redtop grass seedlings, suggested a study of the immediate effect of the antiauxin PCIB on rate of elongation of root hairs.

Methods and Materials

The measurements have been made on root hairs of redtop grass seedlings (*Agrostis alba* L.) by the methods and under the conditions described earlier (Jackson 1960).

Results

That root hairs elongate at a uniform rate for several hours under the control conditions can be seen from Table 1. These results are supported by the earlier findings of Jackson (1959, 1960). During the two hour treatment period, all concentrations of PCIB from 10^{-11} to 10^{-7} M significantly increased the rate of root hair elongation (Figure 1). Compared to the control, the rate was slightly decreased by 10^{-6} and was reduced to about 70 % at 10^{-5} . Elongation stopped immediately at 10^{-4} M. The time course of the response of elongating root hairs can be seen in Figure 2.

Stimulatory effects at 10^{-7} to 10^{-13} M and inhibitory effects at 10^{-6} M were more or less constant over the two hour treatment period, whereas inhibitory effects at 10^{-5} M showed a marked increase after the first hour.

Using the same methods and materials, Jackson (1960) determined the

Table 1. *Effect of various concentrations of p-chlorophenoxyisobutyric acid on rate of elongation of root hairs of Agrostis alba.* Rate of elongation is expressed as a percentage of rate during the 1 hour period immediately prior to application of the test solution. Each point is the average of a minimum of 30 hairs from at least 5 replicate experiments. Standard errors of the means are also given.

Concentration of PCIB <i>M</i>	Rate of elongation, as a percent of pretreatment rate, during various intervals in minutes				
	0 — 30	30 — 60	60 — 90	90 — 120	0 — 120 Min.
10^{-4}	000.0	000.0	000.0	000.0	000.0
10^{-5}	86.8 ± 2.9	80.1 ± 3.1	63.2 ± 3.0	55.0 ± 3.0	71.3 ± 1.8
10^{-6}	95.9 ± 3.7	99.8 ± 3.3	91.0 ± 3.6	91.5 ± 3.7	94.6 ± 1.8
10^{-7}	114.3 ± 3.6	113.7 ± 3.9	111.8 ± 4.5	97.9 ± 4.3	109.4 ± 2.1
10^{-8}	124.9 ± 3.4	129.7 ± 4.3	123.2 ± 4.4	123.6 ± 4.2	125.4 ± 2.0
10^{-9}	112.2 ± 4.3	120.5 ± 5.3	126.8 ± 6.8	129.0 ± 5.0	122.1 ± 2.7
10^{-11}	114.3 ± 3.9	106.6 ± 3.4	109.9 ± 4.3	109.0 ± 4.3	110.0 ± 2.0
10^{-13}	101.5 ± 4.3	106.2 ± 3.2	101.8 ± 3.9	107.5 ± 4.1	104.3 ± 1.9
0 (Control)	102.8 ± 3.4	103.0 ± 2.9	104.4 ± 3.1	100.8 ± 2.6	102.8 ± 1.5

effects of IAA on rate of elongation of root hairs of redtop grass seedlings (Figure 1). All concentrations of IAA from 10^{-15} to 10^{-9} *M* inclusive increased the rate of root hair elongation, with maximum stimulation occurring at 10^{-13} *M*. The results shown in Figure 1 demonstrate that both PCIB and

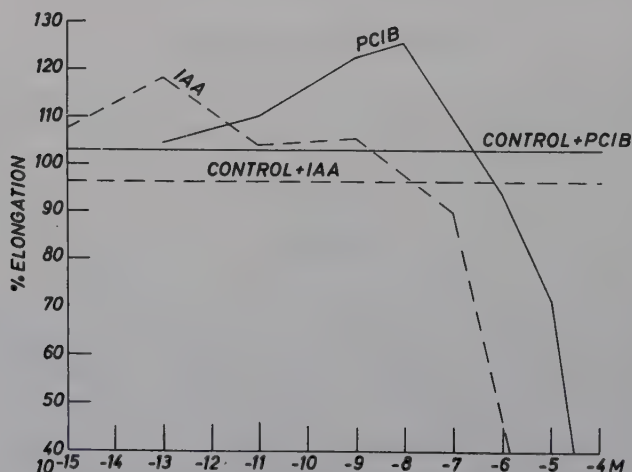
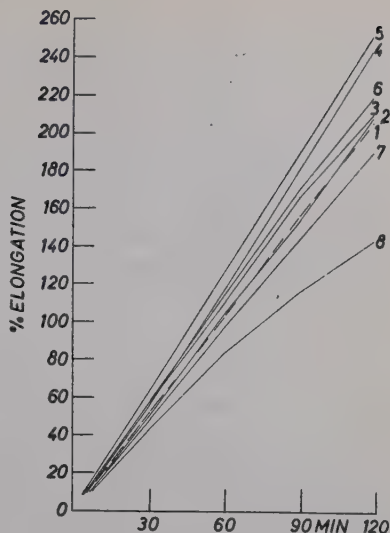


Figure 1. *Effect of p-chlorophenoxyisobutyric acid and indoleacetic acid on the rate of elongation of root hairs of seedlings of Agrostis alba.* The average rate of elongation for the two hour period following treatment at each concentration of PCIB and IAA is expressed as a percentage of the rate of elongation of the same hairs before treatment. The IAA curve is from Jackson (1960). Each point on the PCIB curve represents the average of measurements of a minimum of 30 root hairs. At each concentration of PCIB, 5 to 8 root hairs were studied in each of at least 5 replicate experiments.

Figure 2. Effect of *p*-chlorophenoxyisobutyric acid on the cumulative growth of root hairs of seedlings of *Agrostis alba*. Cumulative growth at each concentration is expressed as a percentage of the growth of these same root hairs for the hour period immediately prior to treatment. At each concentration, 5 to 8 root hairs were measured in each of at least 5 replicate experiments, with a minimum of 30 root hairs. 1 Control, 2 PCIB 10^{-13} , 3 10^{-11} , 4 10^{-9} , 5 10^{-8} , 6 10^{-7} , 7 10^{-6} , 8 10^{-5} M.



IAA significantly increase the rate of root hair elongation and that they increase the rate by about the same amount, *i.e.*, about 125 % of the rate before treatment. However, it should be noted that the concentration of PCIB which causes maximal stimulation is 100,000 times greater than the concentration of IAA required. Therefore, one must conclude that both IAA and PCIB act as "root hair auxins", but that PCIB is weak compared to IAA.

In light of these results, it is of considerable interest to review the effects of PCIB in other growth systems as reported by other workers. A comparison of the effects of PCIB on primary roots, lateral roots, primary leaves, root epidermal cells, and coleoptiles has been made (Figure 3). It is apparent that the majority of experiments have been performed in the range of 10^{-6} to 10^{-4} M PCIB. Over this concentration range, it is without effect or retards the rate of elongation of root hairs of redtop grass seedlings. 9.4×10^{-8} M and 2.35×10^{-7} M PCIB in the presence of 2 % sucrose stimulated elongation of excised primary wheat roots (curve 11). Concentrations as low as 2.35×10^{-8} M were without effect, whereas concentrations higher than 9.4×10^{-7} M were inhibitory. In the presence of 3 % sucrose, PCIB was without effect or inhibitory (curve 12). Lateral roots were not stimulated by any concentration and were inhibited by a concentration of 9.4×10^{-8} M and higher (curves 13, 14). The rate of elongation of intact wheat roots was markedly stimulated by 10^{-6} M PCIB over a period of 2 to 6 days (curves 1, 2), and root epidermal cell elongation rate was increased by 10^{-6} M PCIB acting for 36 and 45 hours (curves 5, 6). Even 10^{-5} M stimulated root epidermal cell elongation

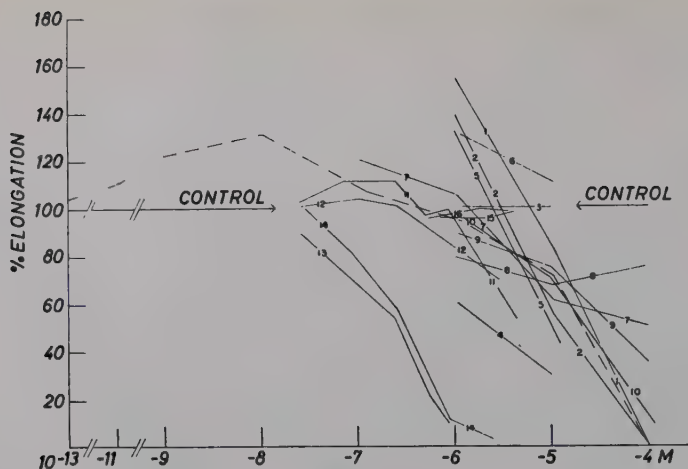


Figure 3. Summary of effects of *p*-chlorophenoxyisobutyric acid on rate of growth of various plant parts. Concentrations of PCIB have been converted to molarity where necessary and rate of growth is expressed as a percent of the control.

Description of Curves

Curve	Reference	Material	Length of Treatment
1	Burström, 1950	Wheat intact primary root	2 days
2	" "	" " " "	6 "
3	" "	Wheat root epidermal cells	10 hours
4	" "	" " " "	18 "
5	" "	" " " "	36 "
6	" "	" " " "	45 "
7	Vitou and Wain, 1959	Cress root intact	2 days
8	" " " "	Maize root segment	2 "
9	Burström, 1950	Wheat, first green leaf	2 "
10	" "	" " " "	6 "
11	Street, 1955	Isolated tomato roots, 2 % sucrose	7 "
12	" "	" " " 3 % "	7 "
13	" "	Tomato lateral roots, 2 % sucrose	7 "
14	" "	" " " 3 % "	7 "
15	McRae and Bonner, 1953	Oat coleoptile sections	12 hours
16	" " " "	" " " "	12 "

during a 45 hour treatment period (curve 6). Rate of elongation of oat coleoptile sections was unaffected by 2.35×10^{-7} M PCIB and was inhibited by higher concentrations (curves 15, 16). The dose-response curve for root hairs, superimposed on this graph, was discussed earlier.

It is suggested that in much of the earlier work on the effects of PCIB on growth of roots, coleoptiles and leaves, the range of concentrations of PCIB has been too restricted and that the lengths of treatment before determining effects on growth have been longer than necessary. The results of the present study suggest that one is not justified in simply characterizing PCIB as an antiauxin alone on the basis of its effects over a narrow concentration range; a range which normally inhibits growth of root hairs. In fact, the results of the present study demonstrate that in certain concentrations PCIB is a "root hair auxin".

Discussion

It is generally accepted that a supraoptimal auxin level normally exists in roots. If this is true, it is of considerable interest to reconcile this with the finding that there is an increase in rate of root hair elongation upon addition of extremely low concentrations of IAA (Jackson 1960), and of low concentrations (10^{-8} M) of the so-called antiauxin PCIB. Burström (1942) proposed that cell elongation in roots occurs in two phases: (1) plastic stretching of the cell wall and (2) cell wall synthesis. He proposed that auxin stimulates the first and inhibits the second phase. Since the second action or phase dominates, added auxin normally retards root growth. Burström (1950) found further support for his theory of auxin action in the results of studies on the effects of PCIB, and of the combined effects of IAA and PCIB, on root growth. PCIB retarded the first and accelerated the second phase of cell elongation. It directly antagonized the inhibiting action of the high concentration of IAA which Burström employed. He concluded that PCIB acts as an antiauxin, perhaps blocking the native auxin, but devoid of all harmful effects in itself.

Åberg (1950) has postulated that upon the addition of a compound such as PCIB, the concentration of effective endogenous auxin may be reduced to a stimulatory level. Several other theories have been proposed which could account for the stimulatory action of PCIB on roots. McRae and Bonner (1953) have postulated that an auxin must assume a two-point attachment at a reactive site in order to be active and that a functional analog (*e.g.* PCIB) of the auxin molecule could inactivate the auxin by competing at the reactive site. This would have the same effect as adding an excess of auxin, since when an excess is added only one point attachments are made.

Wain (1949) and Smith and Wain (1952) have suggested that auxin activity depends primarily on three essential units of structure, *i.e.*, an unsaturated ring-system, a carboxyl group, and at least one α -hydrogen

atom, all having a definite spatial relationship with each other, so that the auxin molecule fits the reactive site. This fit is visualized as a three-point contact of these essential structural units. The possession of all three units in a suitable configuration is necessary for auxin activity.

Tang and Bonner (1947) established that plants possess an active IAA destroying enzyme system, IAA-oxidase. Galston and Dalberg (1954) demonstrated that this enzyme system could be induced by application of IAA and proposed that this may be a general mechanism controlling IAA level in the intact plant. Although they found a tenfold higher oxidase activity in roots than in stems, they demonstrated that young, actively growing regions are very low in oxidase activity. Since a root hair tip, from the time of its inception until almost maturity, is a "young, actively growing region," one might infer that the IAA-oxidase level is low and that its activity could be increased by external application of an auxin or an antiauxin such as PCIB. If the auxin level in root hair tips is supraoptimal, this could explain the stimulatory effects of applied IAA and PCIB. It is difficult to understand, however, why 100,000 times as much PCIB as IAA is needed.

Not only is the mechanism of auxin action not clearly established, but the very terms auxin, antiauxin, and auxin antagonist are difficult to define in relation to their effects on root growth (Torrey 1956, Åberg 1957, and Bentley 1958). In fact, Burström (1955) suggests that the term "antiauxin" should not be used at all in relation to root studies. Hansen (1954) has introduced the term "root auxin" for those compounds which are active as root growth promoters and which also counteract external auxin. Audus and Das (1955) have found that both IAA and certain antiauxins stimulate root growth and suggest that these substances exert identical physiological actions in the same growth system.

Auxins are most rigorously defined on the basis of their ability to induce elongation in shoot cells (Tukey, *et al.*, 1954). If there is a fundamental difference in the way these compounds affect the elongation of shoot cells compared to root cells, the compounds should be defined in relation to the growth system concerned. A "root hair auxin" then is arbitrarily defined as a growth regulator which will increase the rate of root hair elongation. It is obvious that such a substance may act as an auxin and also as an antiauxin. A root hair auxin antagonist would be defined as a substance which is inactive alone, but which counteracts externally added auxin.

On this basis, both IAA and PCIB have been established as "root hair auxins." The maximum increase in rate of elongation of root hairs induced by PCIB and by IAA is almost identical, but 100,000 times as much PCIB as IAA is required. Perhaps the true test of the "antiauxin" activity of PCIB, would be to determine whether it can nullify the stimulatory action of 10^{-13} M

IAA at an equivalent or near equivalent concentration. It is suggested that many of the previous studies of the interaction of PCIB and IAA in roots have been made at inhibitory concentrations of IAA.

Summary

A study has been made of the effects of 10^{-13} to 10^{-4} M *p*-chlorophenoxyisobutyric acid (PCIB) on the rate of elongation of root hairs of *Agrostis alba* L. seedlings. The rate of root hair elongation was increased by PCIB over the concentration range of 10^{-13} to 10^{-7} M inclusive, with maximal stimulation occurring at 10^{-8} M. The rate was slightly reduced by 10^{-6} M, reduced to 70 % of the control at 10^{-5} M, and reduced to zero by 10^{-4} M PCIB. The rate of elongation of root hairs in PCIB-free medium was constant over the two hour treatment period. In contrast to this, the inhibitory effects of 10^{-5} increased markedly during this interval. However, all other concentrations induced their effects rapidly and these altered rates were maintained.

It is concluded that, until a more complete understanding of the mechanism of action is obtained, plant growth regulators should be defined as auxins, antiauxins, etc., only in terms of the system studied. It is proposed that both indoleacetic acid and *p*-chlorophenoxyisobutyric acid be designated as "root hair auxins". Whether PCIB should also be characterized as a root hair antiauxin remains to be determined.

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Preliminary Comparisons on the Facility of Photo-oxidation as Affected by Photoperiodic Induction

By

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The discovery of Parker and Borthwick (1940) that no induction of flowering took place if the leaf exposed to the flowering stimulus was not in contact with an atmosphere containing carbon dioxide and that of Harder *et al.* (1944) that without the presence of carbon dioxide in the night atmosphere flower induction would not take place has led to many studies being carried out on the light and dark fixation of carbon dioxide in relation to photoperiodism (Bode 1942, Spear and Thimann 1954). The experiments of the above authors were carried out using *Kalanchoe* where the dark fixation of carbon dioxide is particularly active. Langston and Leopold (1954) carrying out similar experiments with plants not of the Crassulaceae and therefore not possessing such abnormal powers of dark fixation found also that flowering was inhibited in short day plants when no carbon dioxide was present in the night atmosphere.

Studies of the fate of the carbon atoms fixed from carbon dioxide have been made for dark fixation by Kunitake *et al.* (1957) and for light fixation by Norris and Calvin (1955) and Sen and Leopold (1956). With the experiments on the dark fixation of carbon dioxide only quantitative differences were reported between induced and non-induced leaves. In the light fixation experiments of Norris and Calvin differences were found in the fixation pattern with regard to serine, alanine, and phospho-glyceric acid. As the plants employed by these authors were used when passing from the flowering to the fruiting stage it is doubtful if these differences refer to the induction of flowering but are more probably due to changes brought about by the

senescence of the leaves. Sen and Leopold using plants of a less advanced stage for their comparisons of the labelling pattern in induced and non-induced leaves found no differences except that in many cases there was little or no activity in the sugar phosphate fraction. Nevertheless it was claimed that photosynthesis took place.

It is also known that with increasing light intensities it is possible to reach an intensity where the photosynthesis is inhibited and photo-oxidation takes place (Myers and Burr 1940, Kok 1956). Kandler and Sironval (1959) have shown that in particular there is a reduction in the activity of the light phosphorylation system. Aach (1954) has supposed that the relative ease with which photo-oxidation takes place depends on the state of the chlorophyll pigments. Further Sironval (1957) has demonstrated that the characteristics of the leaf pigment content depends on the day length in which the plants are grown.

In view of these studies connecting photo-oxidation with the state of the pigments and pigment metabolism with the photoperiodic reaction it was thought it would be of interest to measure the extent of photo-oxidation in a plant in the course of photo-induction and to compare this with the susceptibility of photo-oxidation of a plant not exposed to photoperiodic induction. Similarly due to the changes in the pigment content brought about by photo-induction it would be of interest to compare the photosynthetic activity of the induced and non-induced leaves in light of different wave length and intensities.

Methods

Seedlings of *Salvia splendens* var. Feu de la Saint Jean were raised in 8 and 16 hour days (sunlight). When the plants had attained their first nodes (one week after germination), uniform plants were selected and from these the apical bud was removed. It had been shown that even at this early stage (Crawford in preparation) the photoperiodic stimulus had had an effect on the plants; promoting flower induction in 16 hour days, increasing the respiratory activity of the buds and changing the content in chlorophyll and haematin (Sironval, personal communication). The buds from both long and short days were excised in the middle of their day in every case. The excised buds usually 15 in number were placed in rectangular Warburg flasks without a centre well in a $1/100$ M phosphate buffer at pH 4.8. Underneath the flasks were attached the filters necessary for reducing the light to the desired intensity and wavelengths. The filters transmitted red and blue light respectively and in each case transmitted less than 1 per cent of the incident light between 520 and 580 m μ . Thus the light was not of any determined wavelength but was instead a separation of the blue and red ends of the spectrum respectively. Infra-red radiation was negligible as the light only reached the flasks after penetrating 30 cm. of water of the constant temperature bath kept at 27°C. The experi-

ments were carried out with three intensities of red light, one intensity of blue light and with unmasked white light (100,000 lux).

The bicarbonate solution supplying the radioactive carbon was added from the side arm of the flask. In all the experiments the same amount (0.5 ml.) of a standard $\frac{1}{30}$ M bicarbonate solution containing 7 atom per cent C^{14} was used. Illumination was by means of a xenon high pressure lamp from Osram (XBF 6.000). The intensity of the light at the bottom of the flasks was about 100,000 lux. Lower intensities were obtained by attaching wire gauzes to the bottom of the flasks. Photosynthesis was allowed to proceed for 30 minutes. At the end of this time the buds were killed with 80 per cent boiling ethanol and further extracted with ethanol as usual. The buds were then hydrolysed for one hour with 1N hydrochloric acid at 100°C and further extracted with hot water. Aliquots from these extracts were then combusted with potassium chlorate and the radioactivity of the resulting carbon dioxide was measured in the gas phase after the method of Simone *et al.* (1959). Two dimensional chromatograms were made of the alcohol and hydrolysate extracts. The solvents used were those of Bassham and Calvin (1957).

Results

Figure 1 records the relative activity of the ethanol extracts of the buds from long and short days after they had carried out photosynthesis for 30 minutes in the different conditions of illumination. It is seen that the relative

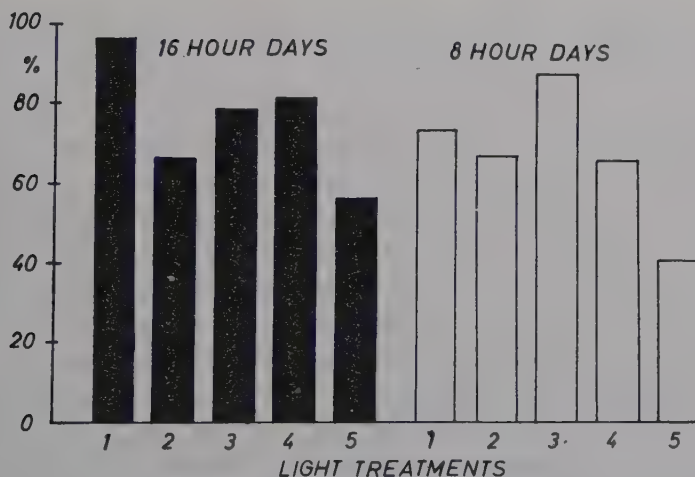


Figure 1. Relative photosynthetic activities of first node buds grown in 16 and 8 hour days as measured in the relative radioactive labelling of the alcohol extracts using light of different intensities and wavelengths. Photosynthesis carried out for 30 minutes in the middle of the day in every case. Light treatments during photosynthetic measurements: 1=blue light; 2=red light — low intensity; 3=red light — medium intensity; 4=red light — high intensity; 5=unmasked white light. On the ordinate per cent relative radioactivity.

Table 1. *Percentage distribution of radio-activity in substances isolated by chromatography from the alcohol extracts of long and short day buds after carrying out photosynthesis for 30 minutes in white light.*

Chromato- gram spot	Substance	Long days		Short days	
		Experiment		Experiment	
		1	2	1	2
1	Sugar phosphates	14.9	15.2	4.4	1.5
2	+	—	1.3	1.3	1.8
3	Sucrose	8.9	16.0	17.7	7.9
4	+	18.1	20.1	17.8	12.1
5	+	8.7	12.8	16.5	53.0
6	Aspartic acid	5.9	3.2	5.1	7.1
7	Glutamic acid	4.4	5.8	6.2	7.2
8	Fumaric acid	1.5	1.1	3.7	—
9	Succinic acid	1.1	2.3	3.8	—
10	α -Ketoglutaric acid	4.2	6.8	5.1	2.9
11	Malic acid	18.7	12.3	15.7	6.2
12	Citric acid	2.2	2.2	1.0	—
13	Glycine	1.7	1.4	—	—
14	+	2.0	2.1	—	—
15	+	7.3	—	—	—

+ = unknown substance.

— = undetectable amount.

activity of the ethanol extracts of respective treatments in long and short days varies but little.

No distinction between long and short day plants could be seen in the fixation pattern of the ethanol extracts as examined by two dimensional chromatograms with the exception of those extracts made from buds exposed to unmasked white light. The percentage labelling of the principal substances in the latter extracts (white light) is recorded in Table 1. It is seen that the different activities in the sugar phosphate region found in this case seem to be related to the pretreatment of the buds with long or short days.

Figure 2 records the relative activity of the hydrolysate extracts. The hydrolysate extract of the buds after two dimensional chromatography was found to contain 94 per cent glucose. It was therefore considered that the total activity of this fraction could be taken as the best measure of the relative photosynthetic activity as it represents the incorporation of C^{14} in the starch reserves of the leaves. Here a very clear distinction exists between the relative activities of the extracts in short and long days for all the wavelengths and intensities used.

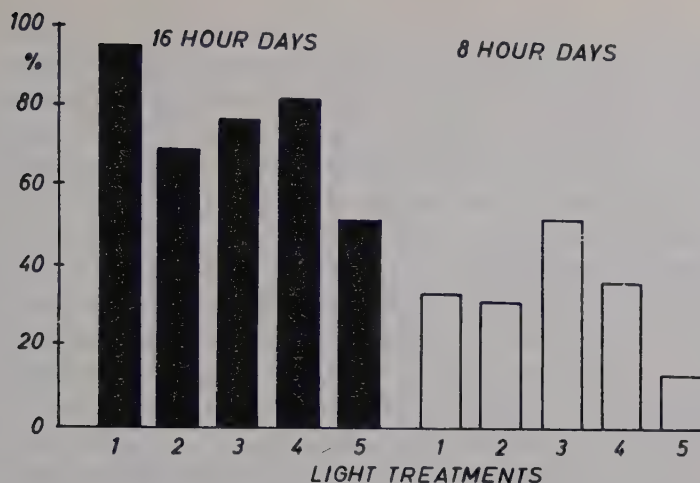


Figure 2. Relative photosynthetic activities of first node buds grown in 16 and 8 hour days as measured in the relative radioactive labelling of the hydrolysate extracts using light of different intensities and wavelengths. As Figure 1.

Discussion

On examining Table 1 compiled from the activity of the chromatogram spots when photosynthesis was carried out in unmasked white light it is seen that the highest activity in the sugar phosphate region with short day buds is 4.4 per cent. The same treatment with long day buds gives values in the region of 15 per cent of the total activity on the chromatogram.

In Figure 2 it is seen that the photosynthetic activity is always greater in the case of the long day plants. However it appears that this is not due only to a greater photosynthetic activity in the long day plants. On examining Figure 2 it is seen that the optimal photosynthetic activity with the short day buds is found in the treatment with red light of medium intensity. Either increasing or decreasing the intensity of the red light reduces the amount of photosynthesis that takes place. In reducing the light intensity this is presumably due to an intensity insufficient to saturate the photosynthetic activity of the plant. Increasing the light intensity reduces that amount of photosynthetic activity presumably for another reason, namely the occurrence of photo-oxidation.

Applying the same argument of the treatment of the buds with white light it could be concluded that photosynthesis is more easily inhibited by the same intensity of white light in the case of the short day plants.

The work of Sironval (1957) on the metabolism of chlorophyll in relation

to photoperiodism suggests a possible explanation for these findings. In plants grown in a photoperiod that is unfavourable for flowering as is the case here for plants kept in short days the chlorophyll metabolism is more labile than in those leaves grown in a photoperiod favourable for flowering. In the former case the chlorophyll-protein linkage is not so strong and therefore would afford less protection for the chlorophyll molecule when it is exposed to strong light.

This changing susceptibility to photo-oxidation could explain the differences obtained in the sugar phosphate labelling in short and long days. This would also agree with the findings of Kandler and Sironval (1959) on the inhibition of light phosphorylation by strong intensities of illumination. It is therefore probable that this difference in the labelling pattern does not represent any fundamental change in the path of carbon in photosynthesis but is instead a secondary modification of the labelling pattern due to the inhibitive effect of intense light.

A comparison of the total fixation of carbon dioxide in long and short days agrees with the findings of Norris and Calvin (1954) and Bode (1942) in that in conditions where the plants flower there is a greater light fixation of carbon dioxide than in day lengths where the plants do not flower. In the experiments carried out with *Salvia* this is most clearly seen in the treatments with blue light (see Figure 2).

In all the treatments except those where photo-oxidation was active the organic and amino acids show approximately the same pattern of labelling. It thus appears that the products of the light fixation of carbon dioxide are metabolised in a similar manner in both long and short days buds. In the experiments of Norris and Calvin the comparisons on the fixation pattern were drawn between leaves of plants already in flower with the leaves of plants that had already passed flowering. It is therefore probable that the changes observed by these authors are not the same as those considered in these experiments using young buds. It could be that the changes in the labelling pattern between the different batches of plants used by Norris and Calvin are due to metabolic changes accompanying senescence.

Summary

Excised buds from young seedlings of *Salvia splendens* var. Feu de la Saint Jean which had been grown in 8 and 16 hour days were examined for their ability to carry out photosynthesis in light of different intensities and wave length. It was known that at this stage in the development of the plant (first node) that the photoperiodic effect of long days had already had some action in promoting flowering.

It was found that the general fixation pattern of the metabolites after 30 minutes exposure to $C^{14}O_2$ was the same for plants grown in long and short days. In short day buds photo-oxidation took place more readily than in long day buds. Photo-oxidation was accompanied by a reduction in the labelling of the sugar phosphates.

In all cases the fixation of carbon dioxide was more active in long days than in short. This difference was most marked in the case of the blue light treatments. For the light intensities used the long day buds made optimal use of the blue light whereas the short day buds used the red, (medium intensity) with maximum efficiency.

These differences in the susceptibility to photo-oxidation as well as the changes in the photosynthetic activity with different light treatments are considered in the light of a possible relation between chlorophyll metabolism and photoperiodic induction.

It was noted that although these differences are secondary when considering the path of carbon in photosynthesis they are nevertheless important for their general effect on the metabolic activity of the plant. In relation to photoperiodism they are considered of special interest as they are differences that occur immediately the plants are placed in different day lengths and at a time when the plants are known to react to the photoperiodic stimulus.

I am indebted to Dr. C. Sironval for advice and discussion throughout the course of the investigation and to Professor O. Kandler for help and facilities with the isotope experiments. The research was supported by a scholarship from the Carnegie Trust for the Universities of Scotland.

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The Relation Between Ion Adsorption on the Cell Wall and Active Uptake

By

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The rapid initial uptake of ions by plant tissues has long been interpreted as due to an exchange or adsorption on the surface of the organ, and the more gradual subsequent uptake as a metabolic movement of these adsorbed ions into the cells. More recently, however, Epstein (1956) points out that "the exchange surfaces or spots are not identical with the entities involved in active transport but the question still remains whether an initial exchange on the exchange surface or spots is a step necessarily preceeding the entry of the ion into the active transport mechanism". Before attempting to investigate adsorption on the cation exchange surface as a rate limiting step in absorption, it would seem desirable to know whether or not these adsorbed ions are available at all for transport into the protoplasm. The following experiments were developed in order to answer this question.

It was earlier shown (Long and Levitt 1952) that ion uptake by potato slices results in a marked accumulation by the protoplasmic particulates. Short term experiments, in fact, indicated that these protoplasmic components may actually contain the "carriers" that first combine with the ions (Chasson 1959). The question put forward here is whether these particulates can capture ions once they are adsorbed on the cell walls.

Materials and Methods

The methods were essentially as described previously (Chasson and Levitt 1957). Potato slices (Russet variety) 2 mm. thick were washed for 48 hrs. in running tap water then dipped for 1—60 secs. into solutions containing Ca^{45} at 3°C. Since adsorp-

tion is a rapid process practically independent of temperature, the cell walls adsorbed considerable quantities of Ca^{45} during this brief dipping (see Table 2). Active absorption on the other hand, has long been known to be undetectable during short periods at this low temperature. The unadsorbed ions were then removed by three successive washes (15 secs., 10 mins., and 10 mins.) in distilled water. All the distilled water used in these experiments was repurified by passing it through ion exchange resin. The slices were then separated into two equal lots. The control lot of slices was immediately placed in 250 meq./l. stable Ca for 5 mins., followed by two 5 min. washes in distilled water. 1700 ml. was used for each wash. The treated lot of slices were rotated (see Chasson and Levitt 1957) for one hour through 1700 ml. distilled water at room temperature, in order to determine whether active absorption from the cell wall could occur. They were then washed three times in exactly the same way as the control set.

Following the final wash the tissue was homogenized in 0.25 M sucrose buffered at pH 8 with phosphate buffer. The gross homogenate was then strained through a double layer of cheese cloth to remove the cell wall debris. The supernate was then layered on a 1.5 M sucrose solution and centrifuged at about $400\times g$ for 2–3 min. in a Servall Angle Centrifuge, Model SS-2. This force threw down the starch grains but the protoplasmic particulates were unable to penetrate the interface. The supernate was then poured off leaving the starch pellet behind. After removal of the starch grains the supernate was centrifuged at $12,000\times g$ for 30 min. to isolate the mitochondria. The supernate from the $12,000\times g$ spin was further centrifuged at $35,000\times g$ for 1 hour, to remove the microsomes. The mitochondrial and microsomal pellets were suspended in distilled water. Aliquots were removed, wet ashed in 1 part 50 % nitric acid plus 1 part 60 % perchloric acid and counted in an SC-16 windowless flow counter.

Nitrogen determinations of the mitochondrial and microsomal fractions were carried out according to the modified Nessler procedure of Umbreit *et al.* (1957).

Discussion of Results

In all eight experiments, the mitochondrial activity increased significantly during the one hour period at room temperature; in six of the eight cases this was true also of the microsomes (Table 1). One complicating factor (Chasson 1959) is the exchange of ions that occurs during homogenization. But this cannot explain the increased activity since the same opportunity for exchange was present in the control set. It may, however, explain the increased activity of the microsomes. For if the mitochondria were the only particulates capable of removing ions from the cell walls, it is still conceivable that some might subsequently be transferred to the microsomes. It is, therefore, impossible to decide from the above data whether one or both of the particulates captures the ions from the cell walls, though they do favor the mitochondria, since in nearly all cases these showed the greater increase.

But do these results actually prove that the particulates obtained their

Table 1. *Uptake of Ca^{45} (cpm/mg.) by mitochondria and microsomes during adsorption and active absorption phases a) in 11 μC N (6.2×10^{-4} M) Ca^{45} , b) in 110 μC Ca^{45} . Activity (Cpm/mg. N).*

Dipping time (Sec. at 3°C.)	Processed immediately after dipping (A)		Dipping followed by one hr. in distilled water at room temperature (B)		B — A (Active absorption)	
	mitochondria	microsomes	mitochondria	microsomes	mitochondria	microsomes
a)						
60	88	77	228	197	140	120
60	144	157	310	183	166	26
15	53	53	91	53	38	0
5	24	25	36	47	12	22
1	14	30	33	32	19	2
1	21	9	44	26	23	17
b)						
1	129	57	256	142	127	85
1	57	70	197	220	140	150

Table 2. Removal of Ca^{45} by washes. Typical results.

Distilled water washes immediately after dipping		Activity (cpm/ml.)	
1		90	
2		11	
3		12	
Control lot (processed immediately)		Treated lot (processed after 1 hr. at room temperature)	
Washes	Activity (cpm/ml.)	Washes	Activity (cpm/ml.)
stable Ca	40	1 hour bath	15
1st dist. H_2O	19	stable Ca	21
2nd dist. H_2O	8	1st dist. H_2O	7
		2nd dist. H_2O	5
Total	67		48

increased activity from the cell walls? It could not be from the external medium, for although the distilled water took on a slight activity by the end of the hour (Table 2) this was only because the diffusion gradient was from the tissue to the medium and not in the reverse direction. It could not be by uptake from free Ca^{45} ions that had diffused into the protoplasm, for if they had diffused in during the brief dipping period, they would certainly have diffused out again during the longer repeated washings. It is true, however, that as long as ions are adsorbed on the cell walls some ions must also be present in the intermicellar fluid of the cell wall. Direct evidence of this is the fact that even after three washes in distilled water, the fourth distilled water bath took on a very small activity by the end of the one hour period at room temperature (Table 2). It is therefore, impossible to decide from these results whether the particulates removed the ions directly from the cell walls, or whether they absorbed them from the extremely dilute intermicellar fluid within the cell wall. But the distinction is immaterial for our purpose. For if the particulates removed the ions from the intermicellar fluid, this must have destroyed the equilibrium and more ions would have to become desorbed from the walls into the intermicellar fluid. That the activity of the cell walls was actually reduced when the particulate activity increased is shown in Table 2. The total activity of the *four* washes from the treated set of slices (48 cpm/ml.) was well below the total for the *three* washes from the immediately processed control set, (67 cpm/ml.). The conclusion is therefore inescapable that *the ions adsorbed on the cell wall are available for uptake by the particulates*, either by direct transfer from the wall or by

desorption from the wall into the intermicellar fluid and uptake from this fluid. In other words, the affinity of the particulates for the ions is greater than the affinity of the adsorption sites on the cell wall for them — a necessary characteristic of the carrier system in the protoplasm, in order for it to absorb ions from very low external activities.

It should be mentioned that a second purpose of these experiments was to determine whether the mitochondria or the microsomes are the site of the "carriers". As mentioned above, the data do not permit a categorical answer, though they do favor the mitochondria.

Summary

1. When potato slices are dipped in dilute solutions of Ca^{45} at 3°C , then washed repeatedly to remove all except the adsorbed ions, the protoplasmic particulates show marked increases in activity during a subsequent one hour period at 25°C .

2. Both the mitochondria and microsomes show these increases, though more consistently in the case of the mitochondria.

3. The activity removable from the slices by washes with stable Ca and distilled water decreases during this one hour period at room temperature.

4. It is concluded that the protoplasmic particulates can actively absorb ions that are adsorbed on the cell walls.

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The Effect of 3-Amino-1,2,4-triazole upon the Metabolism of Carbon Labeled Sodium Bicarbonate, Glucose, Succinate, Glycine, and Serine by Bean Plants

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Since 3-amino-1,2,4-triazole (ATA) was first shown to have phytocidal properties in 1950 much has been learned about its speed of entrance and distribution within plants (1, 4, 6, 12, 16, 17). Relatively little is known, however, about the effect of this substance upon metabolic systems. It was with the thought that such effects must be determined before the nature of ATA toxicity can be fully understood that the present investigation was undertaken.

One of the most conspicuous symptoms of ATA poisoning is the appearance of non-pigmented leaves in the new shoot growth following treatment. Minton *et al.* (15) found the per cent repression of chlorophyll synthesis in terminal bean leaves to be linear with respect to ATA concentrations when the phytocide was applied to the primary leaves. Several workers have speculated about the mechanism of inhibition of chlorophyll formation, but little positive information has been offered. During the current investigation, we have been guided in part by the hypothesis that ATA interferes with the production of porphyrins. And since glycine and succinate serve as the prime precursors for porphyrin synthesis, the metabolism of these compounds in ATA treated plants has been followed.

The effect of ATA upon respiration is not completely uniform with all plants tested. Gas exchange measurements with Canada thistle, cotton, and oats have shown that application of ATA produces an initial stimulation of

respiration followed by a decrease back to normal (9, 10, 14), but no initial stimulation occurred with nut grass (1). Thus far, little information is available about possible alterations in carbon metabolism caused by ATA. In our studies we have attempted to learn how $\text{NaCH}^{14}\text{O}_3$, glucose- U-C^{14} , glycine- 1-C^{14} , glycine- 2-C^{14} , glycine- U-C^{14} , serine- U-C^{14} , and succinate-2, 3- C^{14} are used in the presence of ATA.

Materials and Methods

Excised tips and trifoliates of beans (*Phaseolus vulgaris* var. Black Valentine) were used in all of the following studies. The plants were grown from seed in the greenhouse. In the studies using tips from ATA-treated plants application of a 0.1 M ATA solution containing 0.1 % Tween 80 was made to one primary leaf of each plant on the 7th day after sowing. This treatment resulted in pronounced chlorosis in the terminal growth but did not kill the plants.

Administration of the radioactive compounds was accomplished by placing the tip of 14-day-old plants in small conical glass feeding vials containing an aqueous solution of the compound under investigation. The normal dosage was 1.0 microcurie per tip except in the case of $\text{NaHC}^{14}\text{O}_3$ where the dosage was 12.5 or 25.0 microcuries for treated and normal tips respectively.

After exposure, the stem tips were killed in boiling 80 per cent ethanol, ground and further extracted with hot distilled water. The combined extracts were centrifuged and reduced to an appropriate volume. An aliquot of each extract containing the equivalent of alcohol-water extractable material from about 50 mgs. of tissue was spotted on a large ($18\frac{1}{4}'' \times 22\frac{1}{2}''$) sheet of Whatman no. 1 paper. Chromatograms were developed two dimensionally according to the method of Benson *et al.* (2). Completed chromatograms were exposed to No-Screen X-ray film to locate the radioactive spots. These were then counted with a thin end window Geiger-Müller tube.

Experiment I. In this experiment $\text{NaHC}^{14}\text{O}_3$ was administered to treated and untreated tips in the following manner:

- a) Each of three untreated tips was given 25.0 microcuries of C^{14} in 0.1 ml. of water.
- b) Each of three untreated tips was given 25.0 microcuries of C^{14} in 0.1 ml. of 5×10^{-5} M ATA.
- c) Each of six chlorotic tips from ATA treated plants was given 12.5 microcuries of C^{14} in 0.1 ml. of water.

As the tips took up the radioactive solutions, 0.05 to 0.10 ml. of distilled water was added to wash the vials. After uptake of the wash solution — 4 to 6 hours — the tips were transferred to a dilute nutrient solution containing 1.0 per cent sucrose. All manipulations were carried out in a dark room. The plant tips were maintained in total darkness for one hour prior to the start of the experiment and a dim green light was used at the time the carbon-14 labeled compounds were supplied and at the time of harvest. Temperature was maintained at $23 \pm 2^\circ\text{C}$. Tips were harvested after 6, 12 and 24 hours. At each harvest, one tip each was taken from treatments "a" and "b" and two from "c".

Experiment II. The metabolism of glucose-U-C¹⁴, succinate-2,3-C¹⁴ and glycine-U-C¹⁴ was studied in normal bean tips and chlorotic tips from ATA-treated plants. The labeled compounds were administered in water solutions, as previously described, at the rate of 1.0 microcurie per tip. Duplicate feedings were carried out with each compound. The stem tips were supplied with the radioactive compounds for 4 hours under 600 f.c. of light and then harvested.

The results of this experiment appeared to be at variance with certain preliminary work on the effect of ATA upon glycine metabolism. And when it became clear from subsequent work that no unaltered ATA occurs in the tips of bean plants following application to a primary leaf (4, 16), it became necessary to perform a more comprehensive experiment if any real effect of ATA on glycine metabolism was to be shown.

Experiment III. To further investigate the effect of ATA upon glycine metabolism, a series of bean tips was given the following treatments in the small conical vessels:

Glycine-1-C ¹⁴ in 0.01 M ATA	Glycine-1-C ¹⁴ in water
Glycine-2-C ¹⁴ in 0.01 M ATA	Glycine-2-C ¹⁴ in water
Glycine-U-C ¹⁴ in 0.1 M ATA	Glycine-U-C ¹⁴ in water
Glycine-U-C ¹⁴ in 0.01 M ATA	
Glycine-U-C ¹⁴ in 0.001 M ATA	
Serine-U-C ¹⁴ in 0.1 M ATA	Serine-U-C ¹⁴ in water
Serine-U-C ¹⁴ in 0.01 M ATA	
Serine-U-C ¹⁴ in 0.001 M ATA	

Serine was included because of its close metabolic relationship with glycine in bean plants. All feedings were duplicated and carried out for 24 hours in the light.

The effect of ATA in the supplied solution upon glucose-U-C¹⁴ and succinate-2,3-C¹⁴ utilization was also studied. Feedings with these compounds were carried out for 4 hours in the light.

Results

Experiment I. — An examination of Table 1 shows that the concentration of ATA used did not appreciably affect the percentage of carbon-14 fixed from NaHC¹⁴O₃ into the several substances that became labeled. Apparently the chlorotic tips from ATA-treated plants also fixed radioactive bicarbonate in a manner similar to normal plants. No apparent interruption or stimulation of the respiratory cycle seemed to occur as the result of ATA being present. The slight differences in fixation patterns observed may be attributed to random variation.

Experiment II. — The metabolism of glucose-U-C¹⁴, succinate-2,3-C¹⁴, and glycine-U-C¹⁴ was essentially the same in both normal and chlorotic tips (Table 2). There was a slight accumulation in the chlorotic tips of label from succinate-2,3-C¹⁴ in the amides, glutamine, and asparagine. Also, the lowest percentages of label were found, in sucrose and the phosphorylated sugars

Table 1. *Dark fixation pattern of C¹⁴O₂ in bean plants "Normal" and "Treated" plants received NaHC¹⁴O₃ in water. "Normal & ATA" plants received NaHC¹⁴O₃ in 0.00001 M ATA.*

Compound	Percentage of Total Radioactivity on Chromatogram								
	Normal			Normal & ATA			Treated		
	6 Hrs.	12 Hrs.	24 Hrs.	6 Hrs.	12 Hrs.	24 Hrs.	6 Hrs.	12 Hrs.	24 Hrs.
Aspartic acid	10.0	0.7	0.8	4.6	0.9	0.5	3.3	0.3	0.6
Glumatic acid	4.7	1.0	1.1	2.0	1.8	0.6	2.0	1.0	1.3
Serine	0.9	2.2	0.4	1.0	1.3	trace	1.1	0.7	0.6
Glycine	1.1	—	—	trace	—	—	0.4	—	—
Alanine	0.3	0.5	trace	0.3	0.6	trace	0.3	0.4	0.4
Glutamine	1.0	1.2	0.5	0.4	0.9	trace	3.5	1.0	1.5
Threonine	0.4	2.3	0.7	0.2	0.4	0.4	0.9	0.2	0.6
Asparagine	0.5	5.9	2.0	0.2	1.3	2.2	1.6	1.4	2.4
γ-Amino butyric acid ...	—	—	—	—	0.2	—	—	—	—
Pyrrolidone carboxylic acid	0.4	0.3	0.5	0.6	0.6	—	0.9	0.6	0.9
Malic acid	57.2	81.8	82.5	75.5	79.4	86.7	54.5	73.8	76.5
Citric-iso-citric acid	20.6	3.2	10.0	12.7	9.6	8.2	26.6	18.8	11.8
Succinic acid	0.3	trace	trace	0.4	0.2	trace	0.2	trace	trace
Fumaric acid	0.5	trace	0.6	1.1	0.5	0.3	0.3	0.2	trace
Unknown	2.1	0.7	1.1	1.1	2.0	1.1	4.3	1.6	3.3

Table 2. *Metabolism of glycine-U-C¹⁴, glucose-U-C¹⁴, and succinate-2,3-C¹⁴. The radioactivity in the various compounds is expressed as a percentage of the total in the chromatographed extracts less the activity in the compound fed.*

Compounds Formed	Per cent of total radioactivity					
	Glycine-U-C ¹⁴		Glucose-U-C ¹⁴		Succinate-2, 3-C ¹⁴	
	Normal Tips	Treated Tips	Normal Tips	Treated Tips	Normal Tips	Treated Tips
Aspartic acid	3.4	2.9	3.1	2.8	8.7	4.5
Glutamic acid	5.5	5.5	10.0	5.5	12.0	5.0
Glycine	—	—	2.3	trace	0.0	0.0
Serine	10.0	11.9	2.4	trace	0.0	0.0
Alanine	1.5	trace	2.4	trace	0.0	0.0
Glutamine	1.8	1.4	2.1	3.2	2.5	4.4
Threonine	0.0	0.0	1.6	0.0	0.0	trace
γ-Amino butyric acid ...	2.0	2.0	2.5	trace	3.5	1.2
Leucine-iso-leucine	2.0	1.4	2.4	trace	0.0	0.0
Asparagine	0.0	0.0	0.0	0.0	0.0	4.9
Cysteic acid	2.8	1.8	1.2	0.0	1.9	trace
Proline	0.0	2.2	0.0	trace	0.0	0.0
Sucrose	32.7	35.7	31.5	24.3	12.4	trace
Glucose	trace	trace	—	—	3.8	0.0
Fructose	3.5	2.4	1.8	trace	0.0	0.0
Phosphorylated sugars ...	9.6	7.7	12.5	14.9	5.2	0.0
Malic acid	12.8	5.2	7.5	13.7	32.0	42.4
Citric-iso-citric acid	6.2	10.4	6.9	19.6	16.5	31.6
Succinic acid	1.4	1.5	trace	3.6	—	—
Fumaric acid	1.2	trace	3.6	trace	0.0	1.0
Unknown	3.6	7.0	6.1	8.7	1.4	3.6

Table 3. *The effect of ATA upon the metabolism of glycine-1-C¹⁴, glycine-2-C¹⁴, and glycine-U-C¹⁴ in normal bean tips. Plants exposed to labeled compounds for 24 hours.*

Compound	Per cent of Total Radioactivity ¹					
	— Glycine-1-C ¹⁴		Glycine-2-C ¹⁴		Glycine-U-C ¹⁴	
	0.01 M ATA	H ₂ O	0.01 M ATA	H ₂ O	0.01 M ATA	H ₂ O
Aspartic acid	2.2	trace	1.2	trace	2.0	1.1
Glutamic acid	6.0	3.2	10.6	5.9	11.4	6.6
Serine	6.1	6.6	14.2	22.3	14.2	6.5
Alanine	—	trace	1.3	1.7	trace	trace
Glutamine	2.5	1.4	2.9	2.4	trace	1.0
γ-Amino butyric acid	—	1.1	—	trace	—	trace
Lysine	2.8	2.1	2.7	4.4	2.9	3.4
Asparagine	2.3	—	—	—	trace	1.4
Allantoin	4.9	3.2	9.2	6.3	9.3	7.4
Sucrose	19.9	25.1	15.4	25.2	12.0	18.4
Fructose	3.4	5.0	1.5	6.6	2.3	5.4
Citric-iso-citric acid	3.9	3.4	3.5	2.2	5.2	5.2
Malic acid	6.1	8.0	3.3	4.2	3.7	7.9
Succinic acid	1.9	trace	trace	trace	1.1	trace
Fumaric acid	trace	trace	—	trace	trace	trace
α-Keto glutaric acid	—	—	—	—	—	—
Glyceric acid	16.9	22.1	5.6	3.2	11.2	18.2
Phosphorylated sugars and unknowns	16.7	15.9	16.0	15.4	8.9	17.4
Compound "1"	4.4	—	9.9	—	15.9	—
Compound "2"	trace	—	2.2	—	trace	—

¹ "Total" does not include radioactivity in the compound which was fed.

of these tissues. Such differences, however, were not apparent in the chlorotic tips given either glycine-U-C¹⁴ or glucose-U-C¹⁴.

Experiment III. — The presence of ATA in the supplied solutions produced considerable changes in the glycine-U-C¹⁴ and serine-U-C¹⁴ metabolism by bean tips (Tables 3 and 4) while the metabolism of glucose-U-C¹⁴ and succinate-2,3-C¹⁴ was altered only slightly (Table 5).

With ATA in the solutions taken up by the tips, considerable label from glycine-1-C¹⁴, glycine-2-C¹⁴, glycine-U-C¹⁴ and serine-U-C¹⁴ was transferred to an unidentified, ninhydrin sensitive compound called compound "1" that had previously been observed (4, 16) to be a metabolic product of ATA (Tables 3 and 4). Label from glycine-U-C¹⁴ also entered compound "2", another metabolic product of ATA (4). Significantly, the percentage of label transferred from glycine-U-C¹⁴ and serine-U-C¹⁴ was directly related to the concentration of ATA in the supplied solutions (Table 4). Apparently, ATA forms one or more complexes with glycine, serine or a common derivative.

Table 4. *The utilization of glycine-U-C¹⁴ and serine-U-C¹⁴ by bean tips in the presence of different concentrations of ATA. Plants exposed to labeled compounds for 24 hrs.*

Compound Formed	Per cent of Total Radioactivity ¹							
	Glycine-U-C- ¹⁴				Serine-U-C ¹⁴			
	0.001M ATA	0.01M ATA	0.1M ATA	H ₂ O	0.001M ATA	0.01M ATA	0.1M ATA	H ₂ O
Aspartic acid	1.7	2.0	1.5	1.1	—	—	—	—
Glutamic acid	9.7	11.4	4.8	6.6	13.6	13.4	7.3	12.1
Glycine	—	—	—	—	—	—	—	—
Serine	12.5	14.2	5.1	6.5	—	—	—	—
Alanine	trace	trace	—	trace	1.5	1.6	1.5	1.8
Glutamine	1.3	trace	—	1.0	—	—	—	2.0
Lysine	2.3	2.9	2.1	3.4	4.6	4.6	3.8	5.5
Asparagine	—	trace	—	1.4	—	—	—	—
γ-Amino butyric acid ..	—	—	—	trace	trace	trace	trace	1.1
Allantoin	8.1	9.3	4.5	7.4	7.7	7.4	3.5	7.1
Sucrose	18.0	12.0	5.9	18.4	24.2	17.3	7.8	22.2
Fructose	3.5	2.3	1.5	5.4	2.8	1.0	—	3.5
Malic acid	4.5	3.7	1.6	7.9	3.3	5.7	trace	3.2
Citric-iso-citric acid ...	6.5	5.2	3.6	5.2	3.3	5.1	2.9	4.0
Succinic acid	4.7	1.1	2.5	trace	trace	trace	trace	1.4
Fumaric acid	trace	trace	trace	trace	trace	trace	trace	1.2
α-Keto glutaric acid ...	—	—	—	—	6.5	9.5	5.7	7.2
Glyceric acid	12.2	11.2	9.8	18.2	6.0	5.9	4.7	6.7
Compound "1"	1.7	15.9	47.0	—	3.1	6.1	41.1	—
Compound "2"	1.3	trace	2.9	—	—	trace	3.3	—
Phosphorylated sugars and unknowns	11.9	8.9	7.3	17.4	22.4	20.4	17.6	20.8

¹ Total radioactivity does not include activity in the compound fed.

Discussion

The metabolism of bicarbonate, glucose, and succinate by bean stem tips in the presence of ATA was remarkably similar to the controls. Some indirect evidence was obtained, however, supporting the report of Frederick and Gentile (7) that certain phosphorylation activities may be suppressed by ATA. This was indicated by the reduced labeling from glucose-U-C¹⁴ of sucrose and increased labeling of fructose.

One may hypothesize from the data presented that at least one avenue of ATA phytotoxicity lies in the formation of compound "1". From observations on a number of species (4) this compound is by far the most abundant derivative of ATA produced by the tested plants.

Attempts to identify compound "1" have not yet been successful (5). ATA has been reported to form an iron chelate complex (7) and a glucose adduct (8). It is not at all likely, however, that compound "1" is identical with either of these. When bean seedlings were placed in a solution con-

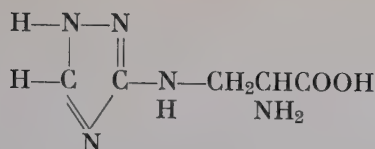
Table 5. *Metabolism of glucose-U-C¹⁴ and succinate-2,3-C¹⁴ in normal bean tips. Feedings carried out for 24 hours in light.*

Compound	Per cent of Total Radioactivity ¹			
	Succinate-2, 3-C ¹⁴		Glucose-U-C ¹⁴	
	0.01M ATA	H ₂ O	0.01M ATA	H ₂ O
Aspartic acid	3.8	3.3	1.6	3.1
Glutamic acid	17.5	16.9	4.7	10.0
Serine	trace	trace	—	trace
Glycine	trace	trace	—	2.3
Asparagine	—	trace	—	—
Glutamine	1.3	trace	1.1	2.1
Alanine	trace	trace	6.3	2.4
γ-Amino butyric acid	3.4	2.7	—	2.5
Proline	trace	—	—	—
Lysine	trace	trace	1.3	—
Threonine	trace	trace	—	1.6
Cysteic acid	—	—	—	1.2
Leucine-iso-leucine	—	—	—	2.4
Citric-iso-citric acid	38.9	38.5	5.6	6.9
Malic acid	20.7	24.5	3.9	7.5
α-Keto-glutaric acid	trace	1.1	trace	—
Succinic acid	—	—	4.9	trace
Fumaric acid	1.2	trace	—	3.6
Glyceric acid	4.7	4.5	4.5	—
Sucrose	1.5	1.6	14.6	31.5
Glucose	—	—	—	—
Fructose	—	—	11.1	1.8
Phosphorylated sugars and unknown	2.6	trace	39.7	18.6

¹ "Total" does not include the activity in the compounds fed.

taining ATA and Fe⁵⁹, compound "1" was not labeled. And, as is shown in Table 5, no label was found in "1" following uptake of glucose-U-C¹⁴ simultaneously with ATA. Furthermore, compound "1" is relatively stable in acid solution (5, 16) a characteristic that would not be expected of a glucose adduct of ATA.

Compound "1" exhibits many of the characteristics of 3-amino-1,2,4-triazolylalanine described by Massini (13). The alanine complex might be hypothesized to be formed from labeled glycine or serine by the conversion of glycine to serine and of serine to alanine via pyruvate followed by linkage to ATA. Or, possibly serine becomes phosphorylated yielding phosphoserine. On the basis of known reactions phosphoserine can be conceived to react with the amino group of ATA and thus yield 3-amino-1,2,4-triazolylalanine of the possible structure:



There is some evidence, however, that compound "1" is not 3-amino-1,2,4-triazolylalanine, or that compound "1" is not formed by the linkage of ATA with alanine. Labeled alanine was not one of the hydrolytic products of compound "1" produced from glycine-U-C¹⁴ (5). This result could be accounted for, however, if the structure shown is correct because the —N—C-linkage is quite stable. Good indirect evidence that alanine is not

incorporated in compound "1" is derived from two experiments. First, root callus tissue of *Pinus clausa* produces large quantities of radioactive alanine from succinate-2,3-C¹⁴ but only a trace from glycine-U-C¹⁴. In the presence of ATA, the pine root callus produced labeled compound "1" from glycine-U-C¹⁴, but not from succinate-2,3-C¹⁴ (3). Second, in the experiments reported here, glucose-U-C¹⁴ furnished a larger percentage of its label to alanine than did glycine-U-C¹⁴, but no label from glucose-U-C¹⁴ entered compound "1" (Tables 4 and 5). Further work is required to provide crucial evidence whether compound "1" is or is not 3-amino-1,2,4-triazolylalanine.

In further speculation about the role of glycine, serine and ATA in the production of symptoms of toxicity it should be recalled that both glycine and serine participate in C₁ metabolism and that there are structural similarities between ATA and several imidazoles. Possibly ATA acts as a C₁ trap and disrupts purine and pyrimidine synthesis. This hypothesis draws some support from Hilton's (11) recent report that L-histidine reverses the inhibitory effects of ATA upon multiplication of certain yeasts.

Inasmuch as there is a sharp reduction in the free glycine and serine pools following the application of ATA, it is likely that at least a portion of the phytotoxic action of ATA can be attributed to interference in the normal metabolism of these compounds.

Summary

1. 3-Amino-1,2,4-triazole (ATA) was found to have very little effect upon the metabolism by bean plants of NaHC¹⁴O₃, succinate-2,3-C¹⁴, and glucose-U-C¹⁴.

2. The effect of ATA upon the metabolism of glycine-1-C¹⁴, glycine-2-C¹⁴, glycine-U-C¹⁴, and serine-U-C¹⁴ was quite striking. In the presence of ATA,

a high percentage of radioactivity from these labeled compounds entered an unidentified compound previously reported to be a metabolic product of ATA.

3. The unidentified compound, compound "1", does not appear to be an iron chelate or a sugar adduct. It may possibly be 3-amino-1,2,4-triazolyl-alanine but this, too, appears unlikely.

4. It is postulated that the phytotoxicity of ATA may result in part from interference in the normal metabolism of glycine and serine.

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Über die Akkumulation von α -Aminoisobuttersäure im Blattgewebe unter dem Einfluss von Kinetin

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Herrn Prof. Dr. Dr. h. c. H. von Guttenberg anlässlich der 80. Wiederkehr
seines Geburtstages gewidmet.

In sich nicht teilendem Blattgewebe bedingt aufgesprühtes Kinetin eine Akkumulation von Aminosäuren. Dabei handelt es sich um Vorgänge eines Transportes gegen ein Konzentrationsgefälle. Wird Kinetin nur einem Teil der Blattspreite eines nicht zu jungen Blattes von *Nicotiana rustica* aufgesprüht, so wird das akkumulative Vermögen so erhöht, daß das Parenchym des Kinetinbezirkes die im ständigen Eiweißabbau anfallenden Aminosäuren nicht nur festhält und damit für eine Eiweißsynthese verfügbar macht, sondern auch dem übrigen Blattgewebe Aminosäuren entreißt. Dieses vergilbt schnell, weil die Resynthese der Eiweiße infolge des Mangels an Aminosäuren nicht mehr möglich ist. Der Kinetinbezirk dagegen bleibt grün; er kann, wenn die übrigen Faktoren der Eiweißsynthese gegeben sind, sogar Eiweiß synthetisieren. Das Verhältnis eines Kinetinbezirkes zu einem Nicht-Kinetinbezirk ist wie das eines jungen Blattes zu einem älteren (1, 2, 4, 5, 6).

Die attraktive Kraft von Kinetinergewebe ist am leichtesten mit Hilfe radioaktiv markierter Aminosäuren zu demonstrieren. Besprüht man den rechten oberen Quadranten eines isolierten Blattes von *Nicotiana rustica* 2 bis 3 mal mit Kinetin (ca. 0,5 μg je cm^2) und trägt links unten in kreisrunder Begrenzung eine stark verdünnte Lösung (0,0185 Mol) von Glycin-[1- ^{14}C] (0,8 μC) auf, so wandert die Radioaktivität des eindringenden Glycins grobenteils schnell zum Kinetinort und wird dort vor allem außerhalb der Nervatur gleichmäßig akkumuliert (Abb. 1). Papierchromatographisch und autoradio-



Abb. 1. *Isoliertes Blatt von Nicotiana rustica*. Rechts oben mit Kinetin, links unten (kreisförmig) mit Glycin- 1^{14}C besprüht (Radioautogramm).

Abb. 2. *Isoliertes Blatt von Nicotiana rustica*. Rechts oben mit Kinetin besprüht, links unten mit α -Aminoisobuttersäure. Vergilbung im Nicht-Kinetinbereich nach 8-tägiger Lagerung (Photo).

Abb. 3. *Dasselbe Blatt wie bei Abb. 2, aber Radioautogramm*.

Abb. 4. *Isoliertes Blatt von Nicotiana rustica mit jungen Adventiwurzeln am Blattstiel und an der Basis der Mittelrippe*. In der Mitte links wurde radioaktive α -Aminoisobuttersäure appliziert.

graphisch läßt sich zeigen, daß Glycin zu einem Teil noch als solches vorliegt, zu einem anderen in Serin verwandelt ist. Ein schwankender Teil der Radioaktivität findet sich auch in anderen Verbindungen, vor allem auch im Eiweiß und in den Nucleinsäuren, beim belichteten Blatt auch in Kohlenhydraten. Andere proteinogene Aminosäuren verhalten sich prinzipiell gleich.

Zur Aufklärung des Mechanismus dieses kinetinbedingten Prozesses erschien es uns erwünscht, die Akkumulation an solchen Aminosäuren zu studieren, die nicht in die Synthese von Eiweiß oder Nucleinsäuren eingehen. Wir wählten zu diesem Zweck α -Aminoisobuttersäure- $^{14}\text{COOH}$ ($680 \mu\text{C}/\text{mM}$). Diese Verbindung wurde bereits von Riggs und Walker (1958) für Untersuchungen über die Akkumulation von Aminosäuren in tierischen Geweben benutzt.

Wir verfahren folgendermaßen: Ein mittelaltes Blatt von *Nicotiana rustica* aus einem beschatteten Nordgewächshaus wurde am 27.6. isoliert und dreimal mit Kinetin (30 mg/l) rechts oben besprüht. Das Blatt wurde bis zum 29.6. in feuchter Kammer bei gedämpftem Tageslicht aufbewahrt und dann links unten mit radioaktiver α -Aminoisobuttersäure versorgt. Der Versuch wurde am 7.7. abgebrochen. Das Blatt ist dann im Kinetinbereich dunkelgrün, im übrigen gelb, jedoch noch turgeszent (Abb. 2). Das Radioautogramm des Blattes ergibt, daß ein wesentlicher Teil der Aktivität vom Applikationsort zum Kinetinbezirk gewandert und dort akkumuliert ist (Abb. 3). Wird dieser Blatteil mit 2,5 % Trichloressigsäure extrahiert, so zeigen löslicher und unlöslicher Anteil ein Verhältnis ihrer Radioaktivität von 70 : 1. Daraus geht hervor, daß die Radioaktivität der α -Aminoisobuttersäure trotz der ungewöhnlich langen Versuchsdauer nicht in die Eiweißfraktion inkorporiert, sondern in der löslichen Fraktion verblieben ist.

Das Radioautogramm eines Papierchromatogramms zeigt die stärkste Aktivität am Ort der α -Aminoisobuttersäure. Eine gewisse Aktivität ist auch im Bereich der Zucker festzustellen, ohne daß damit gesagt sein soll, daß es sich wirklich um solche handelt. Die Substanzmengen sind für eine exakte Ermittlung zu gering. Jedenfalls darf mit Sicherheit gesagt werden, daß die α -Aminoisobuttersäure, ohne in besondere Prozesse der Eiweißsynthese einzugehen, dem Mechanismus der Akkumulation unter dem Einfluß von Kinetin genau so wie andere Aminosäuren unterworfen ist. Der genaue Ort der Akkumulation in der Zelle ist noch unbekannt.

In einem normalen, nicht unter dem Einfluß von Kinetin befindlichen isolierten Blatt wandern die Aminosäuren bevorzugt in den Blattstiel; die Spreite vergilbt. Ist der Blattstiel bewurzelt, so ist eine solche Akkumulation im Stiel nicht zu beobachten und die Spreite vergilbt nicht. Wahrscheinlich werden die in den Blattstiel abwandernden Aminosäuren unter Vermittlung der Wurzeln in die Spreite zurück transportiert (vergl. Mothes u. Engelbrecht 1956). Doch muß auch die Möglichkeit offenbleiben, daß ein von den Wurzeln ausgehender stofflicher Faktor wie das Kinetin die Abwanderung der Aminosäuren aus den Spreitenzellen überhaupt verhindert.

Solche am Blattstiel entstehenden jungen Wurzeln haben in ihren wachsenden Spitzen neue Attraktionszentren für Aminosäuren. Während man aber bisher geneigt war, den Zustrom der Aminosäuren in wachsende Gewebe als Folge ihres Verbrauchs durch die dort ablaufenden Synthesen aufzufassen, wiesen wir darauf hin, daß ziemlich allgemein in solchen jungen Geweben oder Organen eine höhere Konzentration von Aminosäuren vorliegt. Diese akkumulative Kraft jungen Gewebes äußert sich bei der Verwendung radioaktiver α -Aminoisobuttersäure sehr instruktiv. Appliziert man diese einem isolierten Blatt von *Nicotiana rustica* auf die linke Spreitenhälfte, so sieht man die Radioaktivität schon bald danach in den jungen Adventivwurzeln und besonders in ihren Spitzen (Abb. 4).

Es wird damit also bestätigt, daß junge Organe in erster Linie durch einen besonderen Akkumulationsmechanismus ausgezeichnet sind. Die spezifischen Prozesse sind erst eine Folge dieser Akkumulation. Junges Gewebe verhält sich wie Kinetin-Gewebe.

Es ist nicht bekannt, welcher Stoff die Rolle des Kinetins bei der Ausbildung des Akkumulationsmechanismus einer intakten Pflanze übernimmt. Benzimidazol, 2,4-D, Indolylbuttersäure bewirken ähnliche Erscheinungen wie Kinetin.

Summary

Kinetin causes an accumulation of amino acids against a concentration gradient in not dividing leaf cells. Thus kinetin may indirectly cause protein synthesis and growth. Accumulation is not necessarily connected to such a synthesis, because α -aminoisobutyric acid is accumulated in a kinetin tissue as well as in young growing tissues like a proteinaceous amino acid, although it is not incorporated in protein.

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A Study of the Action of Maleic Hydrazide on Processes of Tobacco and other Plants

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Naylor and Davis (1951) showed that high concentrations of maleic hydrazide, *viz.*, 0.005 *M* and above, inhibited respiration of root tips of various species if the pH of the bathing solutions were lowered to 4.0. In view of the relation of maleic hydrazide to maleic acid, they reasoned that a reaction of the compound with sulfhydryl groups of dehydrogenases might occur. Other investigators, Muir and Hansch (1953), and Isenberg, *et al.* (1951) also suggested that a reaction of this type might be the mechanism whereby plant growth is inhibited by the compound. However, investigations by Weller *et al.* (1957) and Leopold and Price (1957) cast strong doubt on this possibility and the idea has continued in disfavor until recently (Hughes and Spragg 1958).

The observation by Isenberg *et al.* that the activities of various dehydrogenases were lowered in MH-(maleic hydrazide) treated onions did not constitute unequivocal evidence for an inhibition of these by the compound, since lowered activity could have been an indirect effect of some more basic disruption of metabolism. Dugnani (1954) reported that various dehydrogenases were inhibited *in vitro* by addition of maleic hydrazide to the reaction mixtures, but the methods of assay for dehydrogenase activity were indirect inasmuch as dyes were used. It therefore seemed desirable, in a study of the action of maleic hydrazide on plant metabolism, to reinvestigate the magnitude and mechanism of respiratory inhibition by MH. The effect of the compound on other processes was also examined.

Materials and Methods

Respiration of Plant Tissues. Axillary buds of tobacco (*Nicotiana tabacum* var. Dixie Bright 101) were removed from field grown plants one week after the plants were topped (flower buds removed). Discs were cut from the bud leaves and, after randomization of these, samples weighing 0.2 to 0.35 g. were placed in Warburg flasks containing 0.1 M K-phosphate buffer, pH 7.2, with or without additions of maleic hydrazide. The tissue was then infiltrated by means of a desiccator and water pump for 30 minutes after which oxygen uptake was measured in the usual way.

Tobacco seeds of the same variety were germinated in the dark on moist filter paper. When the seedlings were 1.5 cm. high, 0.5 g. samples were handled as above except that oxygen uptake in citrate-phosphate buffer at pH 4.0 was also measured.

Also used in these studies were mitochondria obtained from pea (*Pisum sativum*, var. Thomas Laxton) seedlings grown in the dark at 80°F. for 8 days. The seedlings were grown in flats of sand and were used in studies of oxygen uptake with citrate and succinate, and of oxidative phosphorylation with citrate.

Isocitric dehydrogenase. Crude preparations of isocitric dehydrogenase were obtained from snap bean leaves by the method described by Anderson (1956). The assay method involved coupling the dehydrogenation to a plant cytochrome reductase system and measuring the reduction of cytochrome *c* which was dependent on the production of reduced pyridine nucleotide, the latter being the only limiting factor in the system. This method was devised because it was impossible to measure the reduction of pyridine nucleotides at 340 m μ in the presence of the concentrations of MH which were employed. This method was used in assaying the activities of other dehydrogenases in the presence and absence of MH. The reaction mixture in 3 ml. contained in micromoles: Tris (tris-(hydroxymethyl)-aminomethane) buffer at pH 7.4, 150; MnSO₄, 10; TPN (triphosphopyridine nucleotide), 0.22; D,L-isocitrate, 10; cytochrome *c*, 0.30; and FMN (flavin mononucleotide), 0.36. One tenth ml. of the isocitric dehydrogenase preparation and cytochrome reductase extract containing 0.5 mg. protein were included in the mixture. The reaction was started by adding isocitrate and no reaction was detectable in the absence of extract or isocitrate.

Glucose-6-phosphate dehydrogenase. One mg. yeast glucose-6-phosphate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.) was added to the following reaction mixture, the components of which are expressed in micromoles: TPN, 0.2; MgCl₂, 20; cytochrome *c* (oxidized) 0.1; FMN, 0.12; glucose-6-phosphate, 2.0; and glycylglycine, pH 7.5, 17. The mixture also contained cytochrome reductase extract (0.5 mg. protein as measured by the method of Lowry *et al.* (1951) and MH in certain cases. No reduction of cytochrome *c* was observed in the absence of the extract. Some reduction of cytochrome *c* was observed without added substrate, but this was subtracted from the value obtained in the presence of substrate to yield the values presented in Table 2.

Particulate systems. Succinic dehydrogenase activity was measured indirectly by two methods. The first method involved measuring the succinoxidase activity of pea-seedling mitochondria by the uptake of oxygen when succinate was used as the respiratory substrate. Mitochondria were prepared from pea seedlings grown as described earlier by the following method: 40 g. of etiolated pea seedlings were ground at 0 to 4°C with a mortar and pestle in a solution of the following composition: 0.003 M EDTA (ethylenediaminetetra-acetate), 0.05 M Tris Buffer at pH 7.4, and 0.5 M glucose. A small amount of acid washed quartz sand was used to facili-

tate the grinding process. The homogenate was strained through two layers of cheese-cloth and the resulting suspension was centrifuged at $500\times g$. for 12 minutes to remove cellular debris and sand. The supernatant liquid was centrifuged at $15,000\times g$. for 12 minutes, and the resulting supernatant was discarded. The mitochondria were washed twice with 20 ml. portions of the grinding medium and were then resuspended to 3.2 ml. in a solution containing Tris buffer (0.05 *M* at pH 7.4), and glucose at 0.5 *M*. Oxygen uptake and oxidative phosphorylation (as measured by the disappearance of P_i by the Lowry-Lopez method, 1946) were followed in Warburg flasks. The reaction mixtures contained for succinoxidase measurements, the following in micromoles: Tris buffer at pH 7.4, 40; $MgSO_4$, 2.0; ADP (adenosine diphosphate) at pH 7.4, 20; phosphate, 40; and glucose 750. One ml. of the mitochondrial suspension and 0.051 mmol MH (Na-salt) were added. The final volume was 3.2 ml. which included 0.2 ml. of 20 % KOH in the center well. Measurements of oxidative phosphorylation were usually made with certain modifications of the components *viz.*, citrate, 100 μ moles, was used as the substrate and 0.05 μ mole TPN were included.

The second source of succinic dehydrogenase was *Rhizobium japonicum* cells. The method of preparation and assay used was that of Cheniae and Evans (1959).

Malic enzyme. A crude preparation of the enzyme was obtained by grinding 4 g. young soybean roots with a cold mortar and pestle in 6 ml. of 0.05 *M* Tris Buffer at pH 7.4. The homogenate was then centrifuged for 10 minutes at $20,000\times g$. and the supernatant served as the source of enzyme. The reaction mixture (3 ml.) contained as before, cytochrome *c*, cytochrome *c* reductase, FMN, and the following in micromoles: $MnSO_4$, 6.0; Tris buffer at pH 7.4, 61; Tris-malate, 30, and TPN, 0.5. Sufficient quantities of MH (Na-salt) to result in the molar concentrations indicated, and soybean root extract containing 0.16 mg. protein were also added to the mixture. No reduction of cytochrome *c* was observed without added malate or extract.

Diaphorase. An enzyme from tobacco which catalyzes the reduction of dyes was obtained by homogenizing leaves of axillary buds in a mortar containing a solution of the following composition: 0.05 *M* Tris buffer at pH 7.5, 0.0001 *M* glutathione, and 0.001 *M* EDTA. The homogenate was centrifuged at $20,000\times g$. for 15 minutes and the resulting supernatant was fractionated with ammonium sulfate. The fraction obtained between 0 and 40 % saturation was dissolved in 5 ml. of the extracting solution and was dialyzed against a solution containing Tris buffer at pH 7.5, 0.001 *M*; EDTA, 0.001 *M*; glutathione, 0.0001 *M*. This extract was then passed through a column packed with 15 ml. calcium-phosphate gel. The column was eluted with 15 ml. portions of ammonium sulfate ranging in concentrations from 0—10 %. Eluates containing diaphorase activity were used in certain instances and a purified extract from tobacco roots was used in other instances.

The standard assay procedure in this study involved the measurement of cytochrome *c* reduction at 550 $m\mu$ by DPNH catalyzed by tobacco diaphorase and menadione. The complete reaction mixture contained in micromoles per ml.: oxidized cytochrome *c*, 0.06; DPNH (reduced diphosphopyridine nucleotide), 0.3; menadione, 0.030; Tris buffer at pH 7.5, 42; and MH (Na-salt) to produce the concentrations indicated. The quantities of extract added contained from 0.08 μ g. to 0.06 mg. protein. The rate of optical density increase at 550 $m\mu$ due to the reduction of cytochrome *c* was measured with a Beckman model DU spectrophotometer after the reaction was started by adding DPNH. The rate of reduction of cytochrome *c* during the first minute was compared to the rates obtained in the presence of varying levels

of MH. Results obtained using the method of measuring 2,6-dichlorophenolindophenol reduction were similar.

Cytochrome c reductase. Cytochrome reductase was prepared from soybean leaves by the method described by Evans (1956). The crude extract was fractionated with ammonium sulfate and the fraction obtained from 30—60 % saturation was dissolved in 0.05 *M* Tris buffer at pH 7.4 and this served as the enzyme.

Miscellaneous. Cytochrome *c* oxidase and ascorbic acid oxidase from tobacco roots were assayed by conventional methods. Since no effect of MH on these enzymes was observed, actual details of the experiments are omitted. Polyphenol oxidase from potato tubers was also measured in the presence of MH but no inhibitory effect was observed.

Preparation of a nitrate reducing system from *Rhizobium* was made according to the method given by Cheniae and Evans (1959). The experiment with preparation A of table 5 was carried out with the following assay mixture (components expressed in micromoles): potassium phosphate buffer at pH 7.5, 10; NaNO₃ 1.0; menadione, 0.03; DPNH, 0.12 and 1.1 mg. protein from an acetone powder of *Rhizobium* cells.

The assay mixture in the experiment with preparation B was similar except it contained 14 micromoles of succinate instead of DPNH and menadione and 0.1 mg. protein of the particulate preparation.

The autoradiograph was obtained according to the method of Dugger and Moreland (1952). The root tip was taken from a plant to which 5 μ curies C¹⁴-MH (K salt) was applied to the leaves two weeks prior. The same plants were extracted and fractionated for assay of radioactivity associated with the crude protein fractions. The extraction was carried out by homogenizing the treated tissue in 0.05 *M* phosphate buffer in a mortar and centrifuging the homogenate for 15 minutes at 15,000 \times *g*. The supernatant was fractionated with ammonium sulfate and the fractions obtained were washed several times with 10 % TCA, once with 0.1 % KOH, once with 1 % thioglycollate and several times with ether before counting.

Experimental Results

Effect of MH on respiration of plant tissues. Measurement of oxygen uptake by tobacco axillary bud leaves and seedlings in solutions containing maleic hydrazide at a pH close to neutral indicated that only a slight inhibition of respiration resulted over a one-hour period after infiltration (Table 1). Continuing the measurements of oxygen uptake by the seedlings over longer periods of time did not yield evidence that inhibition increased. Naylor and Davis (1951) observed that the pH of the solution bathing the root tips in their experiments had to be lowered to 4.0 before inhibition of respiration by MH was noticeable. The data in Table 1 indicate that after 3.5 hours, the inhibitory effect of MH on respiration at the concentrations employed was substantial when the solutions bathing the seedlings were at pH 4.0.

The rates of O₂ uptake obtained at 210 minutes in the experiment with seedlings at low pH were somewhat higher than during the first hour and this increase had been gradual. However, the rates measured over the period

Table 1. *Effects of maleic hydrazide on the oxygen uptake of young tobacco leaves and seedlings.*

Material	pH	Molar concentration of MH	Time after infiltration (minutes)	O ₂ uptake μl/hr and unit fr. wt.	Per cent inhibition
Seedlings	7.2	0.0	60	202	
		0.009		172	14.8
		0.027		157	22.3
	4.0	0.0	60	80	
		0.027		71	11.2
	4.0 ¹	0.0	270	119	
		0.009		85	28.6
		0.021		63	47.0
		0.027		53	55.5
Leaf discs	7.0	0.0	60	75	
		0.018		63.5	15.3

¹ Oxygen uptake was measured over the period 210—270 minutes after infiltration of the seedlings with the bathing solutions. For details and a discussion of the significance of these observations, see the text.

210—270 minutes were linear. No evidence for contamination by micro-organisms was obtained by microscopic examination of the seedlings and solutions. The measurements were therefore considered valid.

Values for MH residues in seeds whose viability had been destroyed by the compound (Zukel 1957) indicated that concentrations of 0.001 *M* and greater could be expected in tissues. Assuming that the compound would not be distributed evenly throughout tissues, but would concentrate in certain parts of the cell, then actual concentrations much greater than 0.001 *M* might be expected. Reasoning thus, it was decided that in tobacco sucker control the respiratory effect could be one of the causes of growth inhibition and death of the buds. The mechanism was therefore sought. Since several investigators had suggested a reaction with sulfhydryl groups of enzymes, dehydrogenases in particular, a further direct test of this idea seemed important.

Effect of MH on various plant enzymes. The results of experiments in which three soluble dehydrogenases were studied are presented in Table 2. The assay method involved the measurement of cytochrome *c* reduction at 550 m μ , the rate of which was dependent on the rate of production of DPNH by the dehydrogenase. An excess of cytochrome *c* reductase (soybean) and cofactors were present and the dehydrogenation was the only limiting factor in the system. The results show that high concentrations of MH did not produce any inhibitory effects on these dehydrogenases.

The values presented here are means of two determinations and the experiments were repeated several times. Reproducible results were obtained. Pre-

Table 2. *Activity of three dehydrogenases in the presence of malic hydrazide.*

Molar concentration of maleic hydrazide	O.D. change per minute at 550 m μ ¹	Per cent inhibition
Soybean-root malic enzyme		
0.0	0.028	
0.015	0.033	—
0.075	0.032	—
Yeast glucose-6-phosphate dehydrogenase		
0.0	0.028	
0.015	0.029	—
0.050	0.025	—
Isocitric dehydrogenase		
0.0	0.035	
0.015	0.037	—

¹ In the case of yeast glucose-6-phosphate dehydrogenase, some reduction of cytochrome c was observed without added substrate but that amount was subtracted from the total to yield the above values. In the other two cases, no activity was observed without added substrates or extracts.

incubation of the enzymes with MH for periods of time before the reaction was started did not result in inhibition.

The oxidation of succinate by mitochondria, in which succinic dehydrogenase is involved, was not affected by high levels of maleic hydrazide during the interval of time tested (Tables 3 and 5). The results would indicate that not only is succinic dehydrogenase unaffected by MH, but that none of the other components of this particular electron transport system is affected.

In view of the observations by Andreae and Andreae (1953) that MH stimulated the enzymatic oxidation of IAA (indoleacetic acid) and the implication that MH was acting as a redox cofactor, it was thought that the inhibitory effect might be on some oxidase. It had already been shown that

Table 3. *The oxidation of succinate and the oxidation of citrate with concurrent phosphorylation by pea-seedling mitochondria in the presence of maleic hydrazide. The duration of the experiment with citrate was 40 minutes.*

Substrate	MH M	O ₂ uptake μ l/hr and unit N	μ moles O ₂ consumed	μ moles P _i consumed	P/O ratio
Citrate	0.0		11.4	35.0	1.5
	0.005		11.7	36.0	1.5
Succinate pH 7.2 ...	0.0	95.3			
	0.017	96.7			
Succinate pH 5.0 ...	0.0	86.0			
	0.017	78.0			

Table 4. *The inhibition of tobacco-root diaphorase by MH.* The standard assay procedure was used with the amount of MH varied as indicated below. The extract added to each reaction mixture contained 0.08 μ g. protein. — No reduction of cytochrome *c* occurred in the absence of the extract or DPNH, or in the presence of boiled extract.

MH <i>M</i>	O. D. change per minute at 550 m μ	Per cent inhibition
Diethanolamine salt		
0.0	0.117	
0.003	0.105	10.5
0.005	0.097	17.1
0.015	0.083	29.0
0.030	0.063	46.1
0.050	0.048	59.0
0.083	0.030	74.5
Sodium salt		
0.0	0.052	
0.005	0.046	13.5
0.015	0.036	30.8
0.050	0.019	63.5
0.10	0.010	81.0

cytochrome *c* reductase was not affected by MH at high concentrations before the method used to measure dehydrogenase activity could be devised. A subsequent experiment showed that cytochrome oxidase from soybean roots and a solubilized preparation from tobacco were not inhibited by MH. Other oxidases which were not affected by MH were ascorbic acid oxidase of tobacco roots and polyphenol oxidase from potato. No data concerning these experiments are presented.

A diaphorase from soybean leaves was inhibited to about the same extent as was respiration and since the diaphorase was more active than cytochrome reductase in soybean and tobacco, it was thought that the diaphorase might be involved in respiration as an enzyme of the electron transport system. The enzyme from tobacco axillary buds was studied primarily, but a purified preparation from tobacco roots (Sisler and Evans 1958) was also studied. The data concerning inhibition with varying concentration of MH were obtained with the root enzyme and are presented in Table 4. The method of assay used was the reduction of cytochrome *c* with diaphorase, DPNH, and menadione, but the inhibition could be observed using the reduction of 2,6-dichlorophenolindophenol as an assay procedure.

It was also observed that a system from acetone powders of *Rhizobium japonicum* which reduced nitrate using DPNH as the electron donor if menadione or a quinone were present (Cheniae and Evans 1958) was inhibited by MH (Table 5). This suggested that MH might inhibit systems in which

Table 5. *The activity of a nitrate reducing system from Rhizobium japonicum in the presence of MH. A. A menadione requiring preparation from acetone powders of the bacteria. B. A particulate preparation obtained by grinding the bacteria with alumina.*

Enzyme preparation	MH <i>M</i>	$\mu\text{moles nitrite} \times 10^3$ formed per 10 minutes and mg. protein	Per cent inhibition
DPNH			
A	0.0	81.9	— 22.5 41.0 59.8
	0.001	80.8	
	0.01	63.5	
	0.02	48.3	
	0.10	33.0	
Succinate			
B	0.0	40.9	—
	0.05	40.9	

vitamin K is involved. However, the inhibition of the diaphorase was non-competitive with respect to menadione. It was noted also that MH produced no inhibition of oxidative phosphorylation by pea seedling mitochondria (Table 3).

Binding of MH to cellular materials. Concurrently with the study of the MH effect on respiration, attempts were made to find other metabolic systems which were inhibited by MH. One of these was the incorporation of amino acids into protein of pea-root mitochondria by the method described by Webster and Johnson (1955). Although there was no inhibition of this process by MH, it was observed that C^{14} -MH was bound to the proteins in a stable manner. The activity could not be removed by dialysis against salts or water, or by washing the proteins with TCA, KOH, and thioglycollate. This binding was more pronounced with the diethanolamine salt than with the free acid or K-salt.

Table 6. *Radioactivity associated with ammonium-sulfate fractions of leaf and root extracts of tobacco plants which had been treated with C^{14} -MH (diethanolamine salt). The values given have been corrected for background and self-absorption. The values without standard errors were of single samples while the other values were means of two samples.*

Ammonium-sulfate fraction %	c.p.m. per mg. precipitate	
	Roots	Leaves
0 — 30	10.0 ± 2.0	53.0 ± 3.0
30 — 50	13.0 ± 1.0	41.0 ± 2.5
50 — 75	16.0 ± 0.3	28.8
75 — 100	15.0	14.0

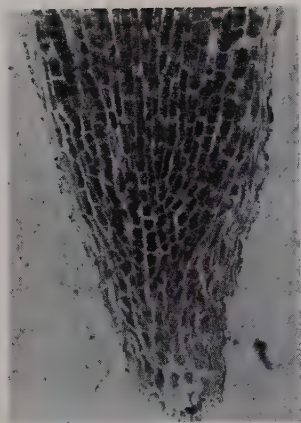


Figure 1. *Photomicrograph of a root-tip section (and underlying autoradiograph) taken from a tobacco plant which had been treated with C^{14} -MH as the K-salt ($\times 128$).*

Crude protein fractions could be obtained by ammonium sulfate fractionation of extracts of leaves and roots obtained from C^{14} -MH treated plants, which had radioactivity associated with them. This association was not shown to be the same as that observed *in vitro*, but it was shown by Stone (1957) that after prolonged extraction most of the radioactivity from treated plants could be recovered as MH. Detailed autoradiographs of root tips indicated that radioactivity was concentrated in the meristematic area, being associated with or incorporated into the nuclei and cytoplasm (Figure 1).

Discussion

This study has shown that MH inhibits oxygen uptake of tobacco tissues, and although the effect is not striking when the pH of the solution bathing the tissue is approximately 7.0, it may be quite pronounced at pH 4.0 in the case of seedlings after a period of 3 to 4 hours following the initial measurement. Measurement of oxygen uptake over this period of time at the low pH value of 4.0, could be open to criticism in that the type of respiration might be changed due to conditions which are injurious to the tissues. Although this possibility existed, the fact that oxygen uptake is inhibited by the compound remained, and presumably this effect was due to an inhibition of some enzyme. The oxygen uptake in the control vessels was somewhat higher between 3 and 4 hours than initially (although the rate was linear over the hour tested) and is probably due to salt accumulation. No evidence of bacterial contamination was obtained by microscopical examination of the solutions containing the seedlings.

The requirement for both low pH and time for a substantial MH inhibi-

tion of O_2 uptake to occur was explained on the basis of the cellular membranes being more permeable to MH at low pH as suggested by Naylor (1951). With increasing time MH was being accumulated in the cells.

Although the respiratory inhibition was considered one of the possible effects of MH which contributed to growth inhibition and death of growing tissues when applied in heavy dosages, *e.g.*, tobacco sucker control and in control of *Oxalis pes-caprae* (Marinos 1958), it was not considered important in explaining the powerful effect of low concentrations on mitosis (Darlington and McLeish 1951) in plants. Rather it seems now that any further attempts to determine the mechanism of that disruption might well be oriented around the antagonism of auxin by MH (Leopold and Klein 1951). This might not be entirely the result of a stimulatory effect of MH on IAA oxidase as suggested by Andreae since the concentration of MH required to stimulate that enzyme *in vitro* was higher than that producing an inhibition of mitosis.

The effect on respiration appeared interesting, particularly since the question of whether MH inhibited dehydrogenases in general had not been satisfactorily answered. It seemed logical to start the search for the cause of the respiratory inhibition by looking at the effect of MH on various dehydrogenases. No effect of MH on isocitric dehydrogenase, glucose-6-phosphate dehydrogenase, or the TPN-malic dehydrogenase was demonstrated. It was also observed that the oxidation of succinate by pea-root mitochondria and particles from *Rhizobium japonicum* was not inhibited by MH under the conditions used. From these results it is apparent that MH is not an inhibitor of dehydrogenases in general by virtue of some property such as strong reactivity with sulfhydryl groups. However, the results do not rule out the possibility that MH does react with sulfhydryl groups in some cases. Hughes and Spragg (1958) have presented evidence that it does although the bond does not seem to be very stable since the sulfhydryl groups can be regenerated by dialysis of the product of reaction between MH and protein.

The inhibition of phosphorylase and β -amylase by MH which they reported, could have been due to a reason other than inactivation of sulfhydryl groups. Certainly evidence for this being the mechanism for the mitostatic action of MH has not been obtained.

After noting that many enzymes were extremely insensitive to MH, it was thought that the MH inhibition of the diaphorase might implicate that enzyme in plant respiration. It can catalyze the transfer of electrons to cytochrome c from reduced pyridine nucleotides providing a compound such as a quinone is present. However, there is no evidence which involves this enzyme in respiration. The respiratory effect could have been due to an MH inhibition of phosphorylase, the reaction Hughes and Spragg (1958) observed later.

The inhibition of the diaphorase was non-competitive with respect to menadione, the vitamin K derivative which was used in this study, and which has been used by investigators who have implicated vitamin K in oxidative phosphorylation and electron transport in other organisms (Brodie *et al.* 1957). If the diaphorase studied is involved in electron transport in any instance, it is not universal, since pea mitochondria oxidized citrate, as well in the presence of MH as in its absence. This indicates that another system, probably a flavin system, is involved in transferring electrons from reduced pyridine nucleotides to the cytochrome system in pea seedling mitochondria. This suggests that the inhibition of diaphorase by MH might be utilized in studying its role since most other enzymes are insensitive to the compound. That MH inhibits nitrate reduction by acetone powder extracts of *Rhizobium japonicum* requiring menadione for activity, indicates that the same diaphorase is involved in that system. However, it should be pointed out that the inhibition was non-competitive with respect to menadione.

The mechanism whereby association of C^{14} -labelled MH with proteins occurs was not elucidated. The possibility that a metabolic product of MH is associated with or incorporated into the salt fractions (proteins and nucleic acids) which were observed was not ruled out. However, since Stone (1957) was able to recover radioactive MH after prolonged extraction of dried plant material, it is believed that the radioactivity is in the form of MH. In view of the report by Hughes and Spragg (1958) that MH reacts with protein sulfhydryl groups, it would be interesting to pursue this "bound" radioactivity further. It might be of interest to note at this point the discovery by Towers *et al.* (1958) that 15 % of the MH in the plants they studied existed in the form of a glucoside. The binding to proteins or nucleic acids may be very important in explaining the action of extremely low concentrations of MH on mitosis.

Summary

This study showed that MH at concentrations of 0.01 *M* and above inhibited oxygen uptake by tobacco tissues to varying degrees, depending on the pH of the solution bathing the tissues. At pH values near 7.0, an inhibition of 10–20 % occurred during the first hour after vacuum infiltration of the tissues by solutions containing MH, while at pH 4.0 the inhibition increased to 30–50 percent. Although the effect is quite apart from the action of the compound on mitosis at very low concentrations, it nevertheless could assume importance when applied in heavy dosage as in tobacco sucker control.

The effect of MH on various plant dehydrogenases was studied by a direct method described here. A general inhibition of dehydrogenases, as postulated by others, cannot be ascribed to MH since none of the ones tested were inhibited.

No inhibition by MH of cytochrome *c* reductase, cytochrome *c* oxidase, and polyphenol oxidase, was observed. An inhibition of a diaphorase by a magnitude similar to the magnitude of respiratory inhibition was demonstrated. That this enzyme is involved in plant respiration was not proved. However, the inhibition of diaphorase by MH should be of importance in studying the function of this enzyme in plant systems. Glucose-6-phosphate dehydrogenase, isocitric dehydrogenase, TPN-malic dehydrogenase and succinic dehydrogenase were not inhibited by high concentrations of MH. If MH reacts with sulfhydryl groups forming a bond between protein and MH stable enough to inhibit enzymatic activity, then it is not clear why all of the dehydrogenase activities tested here were uninhibited.

A binding of MH to proteins and nucleic acids observed in this study was discussed.

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Changes in Metabolic Characteristics of Mitochondria from Soybean Cotyledons During Germination

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The cotyledons of a germinating plant must contain an active metabolic system in order to accomplish the rapid mobilization and export of food materials that are required for growth. The fat, protein, and mineral contents of cotyledons of soybean seedlings aged from one day to several weeks were reported by McAlister and Krober (1951). They showed that disappearance of fat from cotyledons began on about the fifth day of germination, a result which has been confirmed by Kahn *et al.* (1960).

While McAlister and Krober (1951) did not study metabolic changes associated with the gross changes in composition, it may be inferred from their work that during germination there is an increase in the importance of fat as a substrate in the soybean cotyledon.

Since the study of McAlister and Krober (1951) an extensive literature has accumulated to establish the importance of mitochondria in plant metabolism (Millerd *et al.* 1951, Hackett 1959). Mitochondria of seeds or the storage tissues of seeds have been studied by Stumpf and Barber (1956), Akazawa and Beevers (1957), Stanley (1957), Stanley and Conn (1957), and Marcus and Velasco (1960). Mitochondria from hypocotyls of soybean seedlings have been studied by Switzer and Smith (1957) and Key *et al.* (1960). Mitochondria from soybean roots were included by Hanson (1959) in a study utilizing several species. Changes in mitochondria associated with growth were studied by Lund *et al.* (1958) using corn roots.

In view of the importance of mitochondria in electron transport and the concomitant production of metabolic energy (Fruton and Simmonds 1958,

p. 371), it might be expected that changes in composition of the cotyledons would be reflected in and explicable by changes in properties of the mitochondria.

This report concerns changes in the metabolic characteristics of the mitochondria of soybean cotyledons during germination. A preliminary report has been presented previously (Howell 1958).

Materials and Methods

Soybeans (*Glycine max* (L.) Merr.) of Clark and Chief varieties were used.

The following abbreviations are used: ATP — adenosine triphosphate; DNP — 2,4-dinitrophenol; DPN — diphosphopyridine nucleotide; EDTA — (Ethylenedinitrilo) tetracetic acid, disodium salt; SPE — grinding medium consisting of 0.5 *M* sucrose, 0.07 *M* potassium phosphate (pH 6.8), 0.001 *M* EDTA; TCA — trichloroacetic acid; TRIS — trishydroxymethylaminomethane.

Germination. Seeds, lightly dusted with chloranil to retard fungal growth, were germinated between two layers of absorbent paper moistened with 200 ml. of distilled water in 9×12-inch glass baking dishes in the dark at 21°C. The dishes were placed in plastic-enclosed germination chambers approximately 3×1×1 feet in dimensions. After the seedlings had attained a height of 2 to 3 inches the upper layer of absorbent paper was removed and additional water was supplied as necessary. The room was dark at all times except for occasional diffuse light from a single 40-watt cool-white fluorescent lamp, which was turned on only as required by operators servicing the experimental material. No direct light reached the germination trays. An indication of the low light level is the fact that even after a 2-week germination period only minute amounts of chlorophyll appeared in the cotyledons.

Preparation of Mitochondria. At the end of the germination periods, seedlings were harvested and cotyledons were separated from the rest of the plant. The cotyledons were ground in ice-cold SPE grinding medium in an ice-cold mortar or in a Waring blender at —10°C. In some experiments TRIS replaced phosphate as the buffer. In experiments in which both buffers were used, differences in the activity of mitochondria prepared in the two buffers appeared insignificant.

The mash produced in grinding was strained through four layers of cheesecloth and then centrifuged at 8000×*g* for 10 minutes. The liquid phase was recentrifuged at 15,000×*g* for 15 minutes. The precipitate was resuspended in SPE and recentrifuged at 15,000×*g*. All centrifugations were at —10°C. The precipitate from the third centrifugation, suspended in a small volume of 0.5 *M* sucrose, served as the mitochondrial preparation in experimental measurements of oxygen consumption and phosphorylation.

Oxygen Measurement. Oxygen consumption was measured in a Warburg respirometer according to standard procedures (Umbreit *et al.* 1957). The temperature of the water bath was 29°C and the shaking rate was 115 strokes per minute with an amplitude of about 4 cm. Except as noted otherwise, the reaction vessels contained the following materials: 0.25 ml. K_2HPO_4 , 0.1 *M*, pH 6.7; 0.20 ml. sucrose, 1.5 *M*; 0.1 ml. ATP, 14.6 mg./ml.; 0.1 ml. DPN, 33 mg./ml.; 0.1 ml. cytochrome C, 1 mg./ml.;

0.1 ml. coenzyme A, 1 mg./ml.; 0.1 ml. thiamin pyrophosphate, 5 mg./ml.; 0.1 ml. MgSO_4 , 0.02 *M*; 0.1 ml. glucose, 1.1 *M*; 42 units hexokinase; 0.8 ml. mitochondria suspension; 0.4 ml. substrate, 0.2 *M*.

All reactants were kept chilled until added to the Warburg vessel. The center well of each vessel contained 0.2 ml. of 20 % KOH with a 2- \times 2-cm. filter paper serving as a wick. Total liquid volume of the reaction medium was 2.8 ml. and total in the vessel was 3.0 ml.

The reaction period was started after temperature equilibration by closing the 3-way stopcock at the top of the manometer. When DNP was used, it was placed in the sidearm and was tipped in at zero time, *i.e.*, immediately after closing the stopcock. Reaction periods were usually 15 or 30 minutes.

Nitrogen Determination. Nitrogen was determined by nesslerization on an aliquot of the mitochondria suspension estimated to contain between 10 and 100 μg . of nitrogen (Umbreit *et al.* 1957). Following digestion and clearing, Nessler reagent was added and 15 minutes later the optical density was determined at 490 $\text{m}\mu$.

Oxidative Phosphorylation. Oxidative phosphorylation was measured as the difference in inorganic phosphorus content of "initial" and "final" samples. The "initial" sample was taken after temperature equilibration at the beginning of oxygen measurement and the "final" sample at the end of the reaction period. In each case a 1-ml. aliquot was taken and the reaction was stopped by transferring the reaction mixture to 10 % TCA. The phosphorus content of each sample was then determined by the method of Fiske and Subbarow (1925).

Units Used. Oxygen consumption is expressed as $\text{QO}_{2(\text{N})}$, $\mu\text{l. of O}_2$ consumed per hour per mg. of N. Phosphorylation is expressed as P/O, the ratio:

$$\frac{\text{moles of inorganic P converted to organic P}}{\text{atoms of oxygen consumed}}$$

Results

Oxygen consumption by mitochondria from cotyledons of germinating soybeans with the potassium salts of succinic acid, α -ketoglutaric acid, and iso-citric acid as substrates is shown in Figure 1. Succinate was the most effective substrate. α -Ketoglutarate was less efficient and after the fifth day of germination was essentially inactive. By contrast, the effectiveness of iso-citrate increased during the first 5 days and remained essentially constant thereafter through 11 days. Other acids of the Krebs cycle were less active than these three. Mitochondria remained active in the soybean cotyledons for at least 15 days, the longest period studied, as indicated by the effectiveness of succinate as a respiratory substrate over this period. As has been shown previously (McAlister and Krober 1951, Kahn *et al.* 1960), virtually all food reserves in the cotyledons have been depleted by the 15th day after planting.

Relatively low P/O ratios were obtained with succinate, α -ketoglutarate, and iso-citrate as substrates (Table 1), but some parallelism was evident

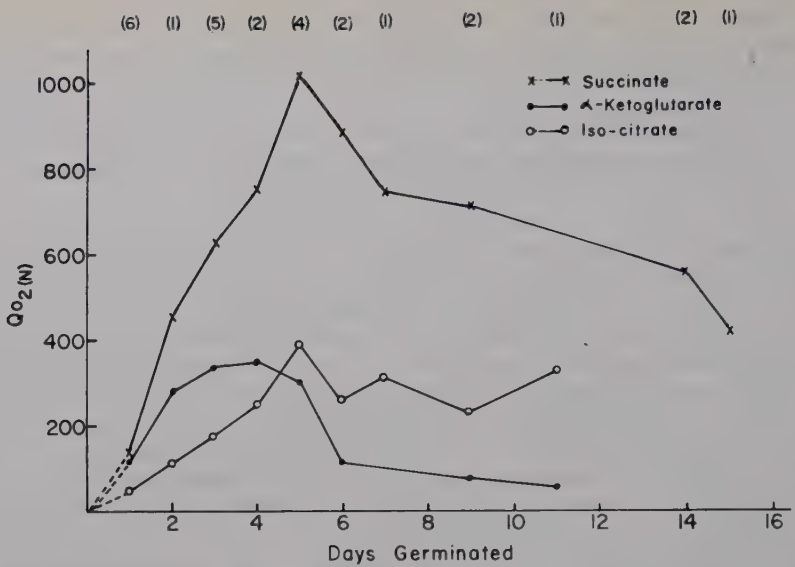


Figure 1. Oxygen consumption by mitochondria from cotyledons of germinating Clark soybeans with substrates as indicated. Numbers in parentheses designate separate experiments contributing to each point. (See text for detailed description of reaction media).

between phosphorylating efficiency and oxygen consumption with succinate and α -ketoglutarate as substrates. Phosphorylation on α -ketoglutarate peaked on the third day and declined substantially after the fifth whereas that on succinate was more nearly constant for 10 days. Even after 15 days phosphorylation on succinate was demonstrable. The P/O ratio on iso-citrate was highest after 1 day of germination and declined slowly thereafter, but after 10 days it was 0.90.

Table 1. Phosphorylation (P/O ratios) by mitochondria from cotyledons of germinating soybeans.

Substrate	Germination period (days)					
	1	3	5	7	9	15
Succinate	0.79	1.48	1.38	1.32	1.17	0.62
α -Keto-glutarate	0.87	1.79	1.26	0.58	0.64	—
Iso-citrate	1.43	1.16	1.10	—	0.90	—

$$P/O = \frac{\text{Moles of inorganic P converted to organic P}}{\text{Atoms of oxygen consumed}}$$

Data for 1, 3, and 5 days are averages of 3 to 9 experiments; data for 7, 9, and 15 days are single experiments or averages of 2 experiments. Chief variety.

Table 2. *Respiration ($QO_{2(N)}$) of mitochondria from cotyledons of germinating soybeans on combinations of substrates.*

Substrate and Quantity/Vessel	Germination period (days)				
	3	4	5	6	9
Succinate (0.02 mmoles)	414	589	524	644	418
Pyruvate (0.02 mmoles)	82	196	62	68	47
Succinate + Pyruvate (0.02 mmoles each)	470	764	760	1018	556
Pyruvate (0.04 mmoles)		108	87	100	
Pyruvate (0.04 mmoles) + Malate (0.001 mmoles) ...		120	115	96	

Chief variety.

Key *et al.* (1960) used pyruvate as a "sparker" substrate for mitochondria from soybean hypocotyls and concluded that a synergistic effect resulted from the simultaneous use of succinate and pyruvate. In the present experiments, pyruvate showed only low activity as a substrate for respiration of mitochondria from cotyledons. The effect of pyruvate and succinate in combination was only additive until about the fourth day (Table 2). On the fifth day a slight pyruvate stimulation of succinate activity was evident and by the sixth day the interaction of these two substrates was substantial. The synergism declined rapidly, however, so that by the ninth day the effects of pyruvate and succinate were little more than additive. Pyruvate alone or in combination with malate, another substance which has been suggested as a sparker of mitochondrial respiration, was a relatively inefficient substrate.

The association of oxygen consumption and phosphorylation can be uncoupled with suitable reagents such as DNP (Fruton and Simmonds 1958, p. 385). The usual effect of DNP is to decrease phosphorylation and increase oxygen consumption, thus lowering the P/O ratio. Mitochondria from ger-

Table 3. *Effect of DNP on respiration and phosphorylation by mitochondria from cotyledons of germinating soybeans. Substrate: Succinate; Chief variety.*

Germination period (days)	Final Concentration (M) of DNP in Reaction Vessels						
	0	6.7×10^{-7}	3.3×10^{-6}	6.7×10^{-6}	3.3×10^{-5}	6.7×10^{-5}	6.7×10^{-4}
			$QO_{2(N)}$				
1	191	186	—	226	98	149	121
3	441	413	424	439	395	366	280
6	616	466	444	406	476	382	258
			P/O				
3	1.48	1.52	1.62	1.41	1.12	1.47	1.63
6	1.12	1.12	1.16	1.13	1.13	1.07	1.12

minating soybean cotyledons did not exhibit the classical response to DNP, as shown by the data in Table 3. Oxygen consumption decreased while P/O ratio remained constant or varied in a random manner as DNP was increased up to a final concentration of 6.7×10^{-4} M. Even the lowest concentration of DNP, 6.7×10^{-7} M, caused a reduction in oxygen consumption by mitochondria from 6-day-old seedlings. There was a tendency for the DNP-induced reduction in oxygen consumption to increase as the seedlings grew older.

Total respiration of the seed is determined not only by the efficiency of the mitochondria but also by the number of mitochondria present. A direct estimate of the number of mitochondria was not available, but the amount of nitrogen in the mitochondrial fraction was assumed to be proportional to the number of mitochondria. Data presented in Table 4 show the amounts of mitochondrial nitrogen and phosphorus per gram (f.w.) of cotyledons after 3, 5, 8, and 11 days of germination. A peak in mitochondrial nitrogen occurred at the fifth day. Phosphorus content of the "mitochondrial fraction" showed a slight peak at the fifth day so that the ratio of nitrogen to

Table 4. Nitrogen and phosphorus content of fractions isolated by differential centrifugation from cotyledons of germinating soybeans.

Germination period and fraction	Nitrogen		Phosphorus	
	μg/g. f.w.	μM/g. f.w.	μM/g. f.w.	N/P
Low-Speed ppt.:				
3 day	110	7.86	1.52	5.17
5 day	126	9.0	.54	16.67
8 day	128	9.15	.22	41.59
11 day	170	12.18	.25	48.72
Mitochondria (15,000×g ppt.):				
3 day	89	6.37	.172	37.0
5 day	172	12.3	.342	35.96
8 day	84	6.01	.275	21.85
11 day	88	6.28	.284	22.43
Microsomes (50,000×g ppt.):				
3 day	269	19.2	1.68	11.43
5 day	161	11.5	.99	11.62
8 day	117	8.35	.59	14.15
11 day	131	9.36	.54	17.33
Soluble:				
3 day	10,885	777	37.5	20.72
5 day	8,530	611	28.8	21.22
8 day	9,110	653	19.5	33.49
11 day	5,970	428	17.4	24.60

Clark variety.

phosphorus (N/P) was about the same on the third and fifth days. Subsequently, however, the phosphorus declined less rapidly than the nitrogen, so a lower N/P ratio was observed on the eight and eleventh days.

Nitrogen and phosphorus contents of various centrifugal fractions are shown in Table 4. Circle (1941) reported that 85 % of the protein nitrogen in mature soybeans is soluble at pH 6.8. An even larger proportion of soluble nitrogen is indicated for the 3-day material in Table 4, but the soluble fraction decreased during germination. Microsomal protein decreased proportionately as much as the soluble fraction, but only a very small amount of the total nitrogen was in this fraction. The nitrogen in the low-speed precipitate, which includes cell walls and other debris, showed some decrease during the germination period. Phosphorus contents of the soluble and microsomal fractions showed changes which were generally parallel to those of the nitrogen, but in the microsomal fraction, phosphorus decreased somewhat more rapidly than did nitrogen. Unlike nitrogen, however, phosphorus decreased in the low-speed fraction by the eleventh day to about one-sixth the initial level. Since during the experimental period nitrogen was increasing in this fraction, a nearly ten-fold increase in the N/P ratio was observed.

Discussion

"Activity" of mitochondria cannot be adequately described except in reference to the substrates used and the age of the tissue. Thus mitochondria from soybean cotyledons may be said to be relatively active ($QO_{2(N)} > 1000$) or inactive ($QO_{2(N)} < 100$) depending on the substrate and age of seedlings used as source material (Figure 1). Similarly, the efficiency of the oxidative phosphorylation system varies with both substrate and age of tissue (Table 1).

The increase in number of mitochondria on about the fifth day of germination has been confirmed in electron-microscope studies by R. F. Bills (unpublished). Since the fractions isolated centrifugally are heterogeneous, an increase in true mitochondria would increase the proportion of metabolically active nitrogen in the so-called "mitochondrial fraction". Such a change in nitrogen composition would cause an increase in oxygen uptake on both total- and unit-nitrogen basis, $QO_{2(N)}$. The increase in mitochondria on about the fifth day probably accounts for the observed increase in respiratory activity observed at this stage of development.

Carpenter and Beevers (1959) have reported the wide-spread occurrence of iso-citritase, an enzyme of the glyoxylate cycle, in the soluble cytoplasm of tissues where active utilization of fat is occurring. The enzyme was absent from species which do not store fat and even from fatty tissues in which

active fat metabolism was not in progress. Marcus and Velasco (1960) showed iso-citritase and other enzymes of the glyoxylate cycle to be present in mitochondria of germinating peanuts and castor bean. The glyoxylate cycle is a key reaction sequence in the conversion of fat to carbohydrate (Kornberg and Beevers 1957). It may be inferred from the present data that the glyoxylate cycle is also present in the mitochondria from cotyledons of germinating soybeans, increasing in activity up to the fifth day, in agreement with Marcus and Velasco. This system appears, or becomes active, at a time appropriate for the utilization of stored fat as reported by McAlister and Krober (1951). The present data, then, considered with those of Carpenter and Beevers (1959) and Marcus and Velasco (1960), provide a metabolic basis for explaining the results of McAlister and Krober (1951).

The failure of DNP to act as an uncoupler is interesting but so far unexplained. Further study is needed in order to interpret adequately this phenomenon in terms of either conventional phosphorylation mechanisms or the particular tissue concerned. It may be related to the cation metabolism of the mitochondria. While several investigators have shown that DNP inhibits uptake of anions and cations in a number of tissues, Chasson (1960) has reported that DNP increased calcium uptake by potato slices.

Summary

Mitochondria were isolated centrifugally from cotyledons of soybean seedlings. The effectiveness of succinate and α -ketoglutarate as substrates for oxygen consumption by mitochondria increased with seedling age up to 5 days. Thereafter the effectiveness of α -ketoglutarate decreased rapidly to a low level whereas that of succinate decreased only slightly and remained at a high level through a 15-day germination period. The effectiveness of iso-citrate increased more slowly but after reaching a peak about the sixth day remained essentially constant. The increase in effectiveness of iso-citrate, coinciding with a decrease in effectiveness of α -ketoglutarate, is interpreted as indicating the appearance of the glyoxylate cycle.

Phosphorylation, as indicated by P/O ratio, reached a higher rate with α -ketoglutarate than with the other substrates, but declined rapidly after the third day. Phosphorylation declined more slowly with the other two substrates. DNP in concentrations between 6.7×10^{-7} and 6.7×10^{-4} M inhibited oxygen consumption but failed to reduce the P/O ratio.

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Induction of Photosynthesis in Etiolated Leaves

By

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1. Introduction. Method and Material

In recent years this laboratory has been engaged in investigations concerning the exchange of carbon dioxide and oxygen which may be observed immediately upon the onset of illumination in experiments with green plants. Several papers on this induction phase of photosynthesis and the associated induction phenomena, especially the occurrence of the primary peak in the CO₂-time curve are published by Vejlby (1958 a, b, 1959 a, b, c, 1960). The measuring of the gas exchange was carried out by means of a modified Noyons diaferometer, in which the thermal conductivity of a gas stream passed over the sample plants or leaves in a plant chamber is compared to that of a by-passing control stream. The method and the modified apparatus is described in detail by Vejlby in the first of the papers listed above.

In the course of the work an idea developed to further elucidate the problems involved in the induction phenomena by comparing the induction taking place in green leaves with the initial phase of photosynthesis in etiolated leaves when these are exposed to light for the first time.

The experiments were made in atmospheric air, or, in some cases, in a specially prepared helium-oxygen gas mixture; to both were added 3 per cent of CO₂. Experimental plants were wheat seedlings (variety Banco) grown at 20°C either in the light in a greenhouse, or in complete darkness. In the diaferometer apparatus the six to eight day old seedlings were placed in a plant chamber designed especially for work with intact seedlings (Figure 1). They remained there in darkness with the gas stream turned on until a thermal equilibrium was attained within the whole system; this required a period of about 30 minutes. Concerning experimental temperatures, see legends of Figures 2 and 3. The dark-grown plants were removed to

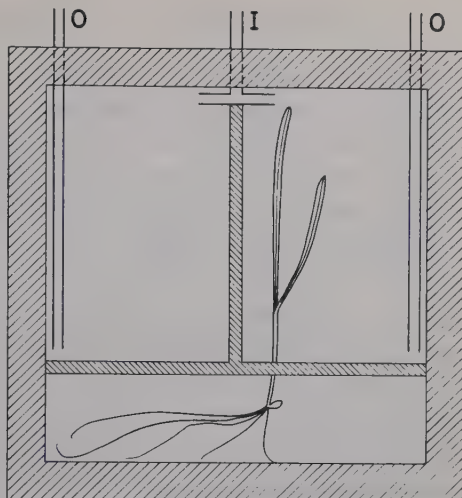


Figure 1. *Leaf chamber (right), control chamber (left), and below root chamber containing nutrient solution. The chambers are arranged in a brass container (inside measures $150 \times 150 \times 10$ mm) with airtight plasticine dividers. I inlet, O outlets for the gas streams. About 20 intact wheat seedlings six to eight days old were used for the experiments; the control chamber contained a comparable quantity of moist filter paper.*

the plant-chamber in a weak green light which had no apparent effect on the conversion of protochlorophyll to chlorophyll-*a*; this was checked spectrophotometrically in ether extracts at regular intervals. A 1500 watt incandescent lamp was used and the intensity received by the leaves was approximately 10,000 lux. Throughout the experiments the diaferometer was adjusted to its maximum sensitivity, *i.e.*, a galvanometer reading of 1 mm corresponds to a change of 0.77 ppm in the carbon dioxide content of the leaf chamber stream.

The investigations has not fulfilled our hopes, so far, of obtaining conclusive evidence of the origin of the induction phenomena. For green wheat leaves we found a photosynthesis-time curve which in every detail, including primary and secondary peaks during the increasing part of the curve, resembled similar curves for grass leaves (*Holcus lanatus*, van der Veen 1949 b), and for many other leaves of flowering plants and mosses (van der Veen 1949 a, Vejlby 1959 b). During the very first illumination of etiolated leaves with white light from the lamp (referred to in the following as initial illumination) we found gas exchange time curves which strongly differed from the time curves of green leaves. The results appear to disagree in more than one respect with our knowledge so far of the initial stage of photosynthesis following the first exposure of dark grown plants to light and the resulting onset of chlorophyll formation.

Older and recent literature concerning the development of the photosynthesizing mechanism in etiolated plants have been reviewed in detail by James H. C. Smith a few years ago (1954). Hence in the following we need quote only papers directly relating to our experiments.

2. Experiments in Continuous Illumination. Discussion

Figure 2 presents a typical result obtained by directly exposing etiolated plants to an intensity of 10.000 lux. Until the fifth minute when illumination begins, the course of the time curve is even (here corrected for zero-point drift of the diaferometer and adjusted to follow the axis of abscissas, see Vejlby 1958 a) showing a steady formation of respiratory carbon dioxide. Immediately following light exposure the time curve descends steeply, the thermal conductivity of the gas leaving the leaf chamber being reduced as compared to that of the control stream. The reduction having attained its maximum value 2 to 3 minutes after the onset of illumination, this value is then maintained with only minor fluctuations throughout the duration of the light period. We have no knowledge of the reason for the fluctuations, and they do not occur in all experiments. When the light is switched off, the time curve ascends steadily until the level (the zero-point slope) observed before exposure to light is reached. The slope of the corrected time curve immediately following the onset of light and immediately following its discontinuation is largely determined by inertness of the plant reactions, although a certain inertia in the response of the apparatus cannot be excluded as a contributory cause.

Theoretically, the reduction observed in the thermal conductivity of the gas stream after its passage through the leaf chamber may have three causes, *i.e.*, 1) an increase in water vapour pressure, 2) an increase in the carbon dioxide content, or, 3) a diminution of the oxygen content of the gas.

1) The heating to which the leaves are exposed through illumination may cause an increase in evaporation affecting the thermal conductivity in the said way (the conductivity of water vapour is somewhat lower than that of atmospheric air, Laby and Nelson 1929). Measurements by means of thermoelements showed the raise in leaf temperature caused by illumination to be a mere 0.05°C and the air surrounding the leaves showed a similar increase in temperature. The comparative insignificance of the increase in leaf temperature is understandable in view of the fact that the main part of the infrared emission from the lamp is trapped in layers of water in front of the leaf chamber. Although the temperature increase observed could hardly be assumed to have any great effect on the measurements, every conceivable precaution was taken to ensure that the temperature and the water vapour concentration of the two gas streams were identical (we aimed at complete water vapour saturation in leaf chamber stream and control stream). One of the alterations introduced to better the experimental arrangement consisted in passing the control stream through a chamber, identical in dimensions to the leaf chamber, and exposing both of the chambers to light simul-

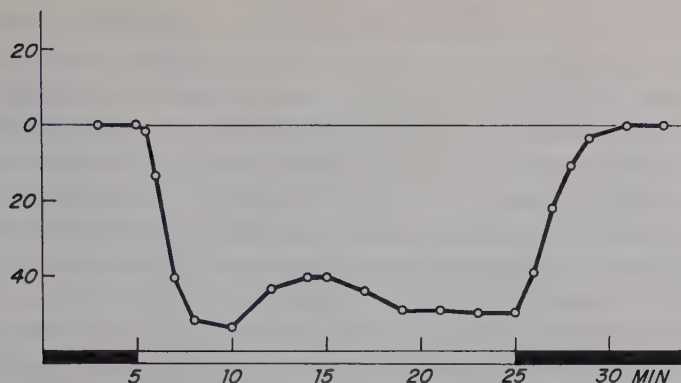


Figure 2. Time curve for carbon dioxide formation during photo-oxidation in etiolated wheat seedlings. The dark-grown plants were exposed to protochlorophyll/chlorophyll-transforming light (incandescent lamp, 10,000 lux) for the first time in the 5th minute counted from beginning of measurements. Temperature of the air stream 15°C. Illumination is discontinued after 25 minutes. Ordinate: galvanometer readings (mm).

taneously (Figure 1). The curves in Figures 2 and 3 represent measurements made with the thus modified apparatus.

It appears reasonable on the background of the adduced arguments to exclude the possibility of changes in the water vapour pressure influencing the course of the time curve in Figure 2.

2 and 3) Excluding the first theoretical possibility the reduction observed in the thermal conductivity of the leaf chamber stream can be due only to a formation of CO_2 from the etiolated leaves. A simultaneous consumption of oxygen would act conductivity-reducing in a similar way, but in the case of the gas mixture used in the experiment (atmospheric air enriched with 3 per cent CO_2) the effect on the net result would be but slight since there is no great difference between the respective thermal conductivities of oxygen and of atmospheric air (conf. Aufdemgarten 1939 and Laby and Nelson 1929). A determination of the consumption of oxygen by etiolated wheat seedlings exposed to light for the first time in a helium-oxygen-carbon dioxide mixture (giving possibilities for simultaneous measurements of oxygen and carbon dioxide exchange; for the method see Vejlbj 1959 a), gave an unequivocal result: an uptake of oxygen took place simultaneously with the formation of carbon dioxide, starting at the onset of illumination and ceasing immediately after its discontinuation. Unfortunately, it was impossible with the present apparatus to measure the absolute amounts of carbon dioxide and of oxygen. Except for the fact that they occur simultaneously nothing is known about the interrelationship between the uptake

of oxygen and the evolution of carbon dioxide in the experiment recorded in Figure 2.

The exchange of carbon dioxide for oxygen registered seems to suggest an increased respiratory intensity. In this connection the temperature rise in the leaves during exposure to light is suggestive, but an increase of a mere 0.05° could hardly be expected to affect the rate of respiration to any great extent. One might next imagine a light induced increase in respiration such as the one registered in etiolated barley seedlings by Weintraub and Johnston 1947 (see also Weintraub 1944); but this type of increase does not occur momentarily immediately following exposure to light, and it disappears only gradually in the course of some hours after the discontinuation of the illumination. The fact that the duration of the increase in gas exchange is identical with the period of illumination appears to indicate that the greater part of the carbon dioxide evolution (and oxygen consumption) measured at an initial illumination of etiolated plants is due to a photo-oxidation process. The mechanism responsible for this reaction is unknown. Photo-oxidase, which is present in preparations of spinach leaf chloroplasts (Neiman and Vennesland 1959) is absent in etiolated grass seedlings (Bishop, Nakamura, Blatt and Vennesland 1959).

Hence under our experimental conditions no photosynthesis could be demonstrated to take place during the initial illumination of etiolated seedlings; in its stead, a photo-oxidation occurs, continuing at an unreduced rate for 20 to 30 minutes. We were unable to follow up the phenomenon for periods of longer duration due to the fact that the zero-point of our diaferometer tends to drift rapidly when the apparatus is adjusted to a high degree of sensitivity. Experiments in which higher and lower light intensities followed each other during initial illumination of etiolated seedlings showed the rate of formation of carbon dioxide to depend upon the light intensity: the higher the light intensity, the greater the carbon dioxide output.

In 1910 Miss Irving in the laboratory of F. F. Blackman measured the output of carbon dioxide from etiolated barley plants during initial illumination with continuous light as in the present experiments. In plants at the same stage of development as ours no photosynthesis was observed during a period of 14 hours of continuous illumination. Only at a repeated illumination following a subsequent dark period of 10 hours was a reduced output of respiratory carbon dioxide observed indicating the onset of photosynthesis. The experiments were made in streaming atmospheric air. Young plants (coleoptile stage) appeared to require 2 day periods and 2 night periods before photosynthesis could be observed. No photo-oxidation was observed probably due to the fact that only weak light from a paper-screened north-facing window was used.

The technique used in other studies where the induction of the photosynthetic process in etiolated plants was observed from the very first moment of illumination (Inman 1935, Smith 1954, Tolbert and Gailey 1954, etc.) did not allow observation of any possibly present initial photo-oxidation reaction.

3. Experiments with Light and Dark Periods of Varying Lengths. Further Discussion

Some of our results from experiments with discontinuous illumination are presented together in Figure 3. The experiments were arranged in such a way that seedlings having no chlorophyll, after a brief period of illumination, were illuminated now and again for a short time with intermediate dark periods of from 2 to 10 minutes, in order to determine whether or not photosynthesis was initiated. The changes from dark to light and vice versa were effected by means of a movable screen placed between the light source and the plant chamber.

Curve A shows a typical result. As is the case in the other curves, only a number of the measurements were plotted, since for technical reasons it was impossible to include all of the determinations made at 15 second intervals. Initial illumination induces a strong photo-oxidation continuing throughout the two minutes when illumination is maintained. At renewed light exposure after 10 minutes of darkness photo-oxidation again sets in, but the rate of carbon dioxide output during the light period is slightly lower than that found during the initial illumination. Third and fourth periods of illumination (after 24 and 36 minutes, respectively, counted from the first moment of exposure to light) show decreasing rates of output. During the fifth period of illumination it is possible after 48 minutes to demonstrate an uptake of carbon dioxide which may be considered a distinct indication of the onset of the photosynthetic process. During the next period of illumination (after 60 minutes) the rate of photosynthesis has increased.

A similar picture results when the light periods are reduced from 2 minutes to 30 seconds (curve B); the output of carbon dioxide decreases from one period to the next during the first four periods, while during the fifth it is possible after 48 minutes to demonstrate an uptake of carbon dioxide. A reduction of the light periods to five or three seconds (curve C) excludes the possibility of observing photo-oxidation, but it is possible to demonstrate photosynthesis during a five minute period 50 minutes after the first flash of light.

At the first discussion of these data we want to emphasize the fact that uptake of carbon dioxide by photosynthesis (*i.e.*, true photosynthesis, since

the axis of abscissas represents the rate of respiration) may be demonstrated unequivocally to take place in the wheat leaves after 48 to 50 minutes from the onset of intermittent illumination. Within the experimental conditions used, this period of time appears to be independent of the amount of light supplied, a total light period of 8 minutes giving the same result as one of only 15 seconds. The length of the dark periods on the other hand, may affect the result: a reduction of the intervals between light periods of the same length (2 minutes) from 10—12 minutes to 2 minutes (Figure 3, compare curve A and curve D) reduces the period before an uptake of carbon dioxide is demonstrated from 48—50 minutes to 36 minutes (the reduction may be even greater, but the measurements constituting curve D do not all appear sufficiently reliable). In this connection it may be noted that the extension of a dark period increase the duration of time until photosynthesis may be observed through uptake of carbon dioxide. In the experiments on which curve E (Figure 3) is based a preliminary treatment of 2 minutes of illumination followed by 40 minutes in the dark preceded the recorded measurements. Thus the length of time from the initial illumination until the first peak above zero level (respiratory level) may be observed is increased to 72 minutes. The duration does not change significantly when the preliminary 2 minute light treatment is replaced by a 5 minute exposure (curve F); this represents a second example of the fact that the inducement of photosynthesis does not to any great extent depend on the quantity of light supplied as long as this reached a certain minor value.

This is the extent of our results so far. They appear to establish the fact that the organization and inducement of the photosynthesis mechanism in etiolated, not photosynthetically functioning plants are promoted by exposing them to alternating periods of light and dark. Hence our results are in agreement with observations published by Smith in 1954. Smith found that two 5 minute intervals of illumination separated by a 110 minute dark period increased the photosynthetic ability of etiolated barley seedlings to a greater extent than did one 10 minute period of illumination followed by 110 minutes of darkness. On the basis of his experiments Smith concluded that the increased photosynthetic capacity was due to ordinary metabolic processes

Figure 3. *Experiments in intermittent light with etiolated wheat seedlings which have not previously been exposed to protochlorophyll/chlorophyll-transforming light.* Ordinate: galvanometer readings chiefly representing production or uptake of carbon dioxide, in mm. Illumination and dark intervals are indicated on the axis of abscissas in white and black. A, 2 min. light, 10 min. dark; B, 30 sec. light, 11.5 min. dark; C, 3 sec. light, 10 min. dark repeated 5 times, then subsequently 5 minutes light; D, 2 min. light, 2 min. dark; E, 2 min. preliminary light+40 min. dark, then experiments using periods of 2 min. light and 8 min. dark; F, 5 min. preliminary light+40 min. dark, then experiments in periods of 2 min. light and 8 min. dark. A, B, D, E, and F at 20°C, C at 24.4°C.

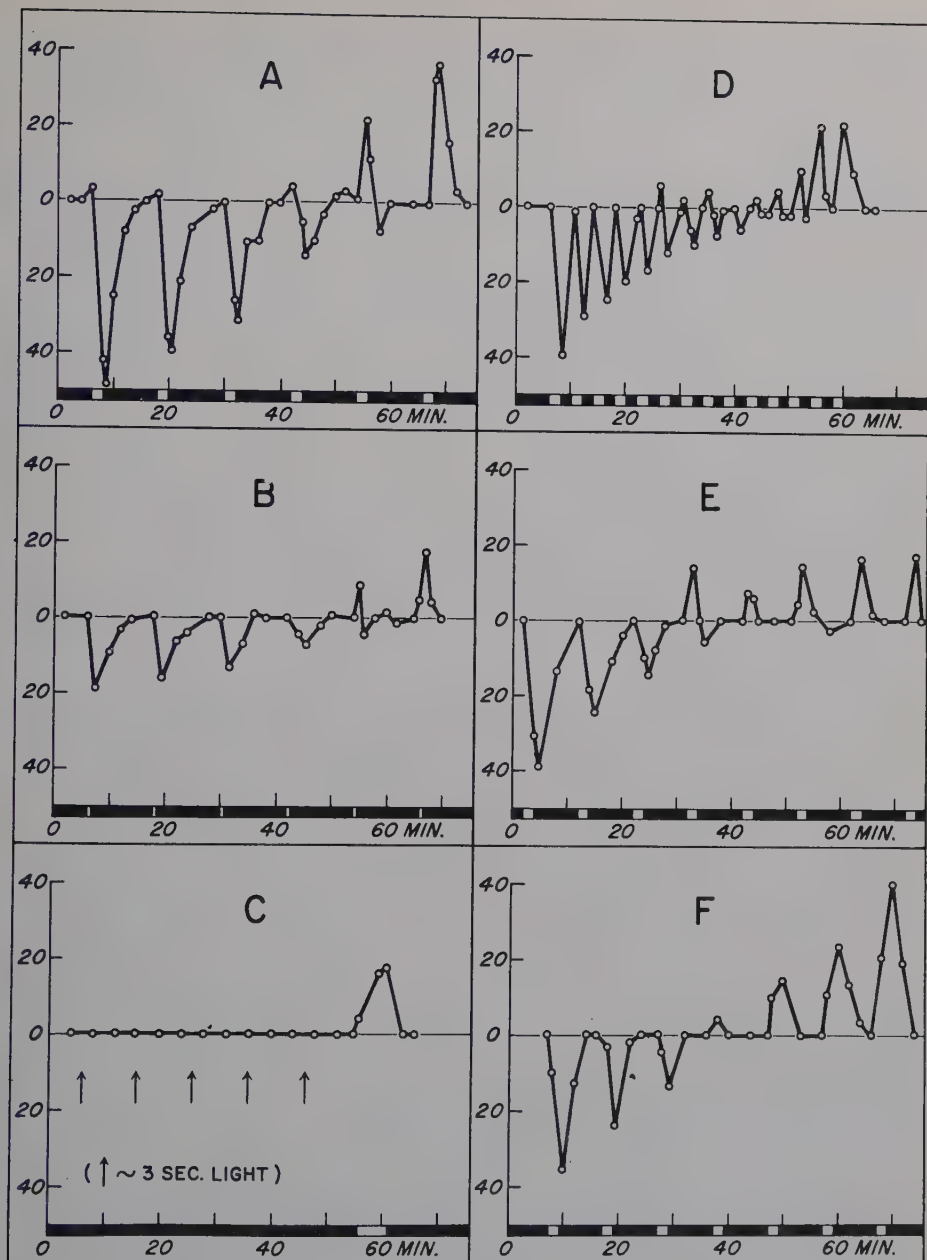


Fig. 3.

(effect of the dark period) as well as to photochemical reactions (effect of light period no. 2). The present authors agree with Smith's conclusions; the present study demonstrates further that the duration of the light and dark periods need not necessarily be as long as those used by Smith in order to produce effects.

The previously quoted results of Miss Irving appear to suggest that light period, dark period, and light period again must necessarily succeed each other in the order named to ascertain the inducement of photosynthesis. Other experiments in which Miss Irving exposed etiolated shoots of barley and bean plants to artificial light (gas light) gave no positive results within 60 hours including 3 light periods. She even failed to demonstrate any photosynthetic activity in plants in which the formation of chlorophyll had proceeded to the extent that the leaves were grass green. These negative results may possibly be due to a too low content of carbon dioxide in the gas used for the experiments. For the experiments in gas light Miss Irving used streaming CO₂-free air and photosynthesis could be demonstrated only by reassimilation of respiratory carbon dioxide. It is known that a low carbon dioxide concentration can occasionally reduce, or even completely inhibit the photosynthetic process in leaves (Gabrielsen 1948, 1949, Gabrielsen and Schou 1949).

Studies by other authors suggest that the light, dark, light sequence is not essential for the development of the photosynthetic mechanism. Inman 1935 studied etiolated seedlings of corn, wheat, and oats in continuous light and was able to demonstrate a formation of oxygen after an average exposure duration of 150 minutes. Using continuous illumination Tolbert and Gaily 1955 (see also Tolbert 1957) found that photosynthesis in etiolated wheat plants (fixation and incorporation of ¹⁴CO₂ in photosynthetically formed products) started only after a period of about 5 hours. On the basis of these results it appears permissible to conclude that the light, dark, light sequence is not necessary for the inducement of photosynthesis but that the results of Smith and of the present authors show merely that the reactions leading to the development and activation of the photosynthetic mechanism are accelerated by the use of intermittent light.

In the plants used by Irving, by Inman, and by Tolbert and Gailey the formation of chlorophyll was quite advanced and visible to the naked eye by the time it was possible to demonstrate photosynthetic activity. Hence the results of these authors are in contrast with our observations in which photosynthetic activity could be demonstrated in terms of uptake of carbon dioxide long before it was possible to detect any traces of chlorophyll by changes in the initial yellow colour of the wheat leaves. Probably the photosynthetic activity which we observed was brought about solely by means of

the amount of chlorophyll-*a* formed by illumination of the protochlorophyll accumulated in the etiolated leaves. We found this conversion to take place very rapidly, most of the protochlorophyll having been converted into chlorophyll-*a* after 1 second of light (methods, see Madsen 1960). According to Shibata (1957) and Virgin (1960) no new formation of protochlorophyll can be observed in wheat, corn, and bean leaves until about 30 minutes after an initial 1 minute illumination had converted the primary, dark formed protochlorophyll (P 650).

Observations published by Blaauw-Jansen, Komen, and Thomás, 1950, form a complete contrast to the studies discussed so far. These authors found that leaves of dark grown wheat seedlings contained a minor amount of chlorophyll-*a*, and that they, when transferred to a Warburg vessel, distinctly showed photosynthetic activity (oxygen formation) after a 40 minute dark adjusting period. This may possibly have been due to erroneous use of too strong, green light during the preparation of the experimental material as the authors themselves suggest. As stated before, no trace of chlorophyll-*a* could be demonstrated in any of our plants before they were exposed to white light in the plant chamber of the diaferometer.

In the discussion we have so far assumed that the first observed true photosynthesis is registered by a peak in the time curve representing an uptake of carbon dioxide. However, a photosynthetic reaction may in fact have occurred even before the appearance of this peak.

Curves A, B, D, E, F in Figure 3 all show a diminishing formation of photo-oxidation carbon dioxide during the light periods following the first dark period of each experiment. It appears not unreasonable to assume that a constant photo-oxidation in this case is compensated by a gradually increasing photosynthetic activity. If so, this activity would commence already after the first light + dark period and increase in strength after the following exposures to light and dark.

This explanation is contradicted, to a certain extent, by Shibata's investigations on the stepwise conversion of chlorophyll-*a* in intact etiolated leaves subjected to illumination for the first time (1957). Here it was possible to show that immediately after its formation by illumination of protochlorophyll, the chlorophyll appears with an absorption maximum at λ 684, changing subsequently to a maximum at λ 673, and finishing up with a maximum at λ 677 m μ . If then photosynthesis can take place only by means of the chlorophyll-protein-compound normally occurring in green leaves and which has its absorption maximum at λ 677 m μ , Shibata's results (with bean and corn leaves) show that this compound does not develop until 30 to 60 minutes after the initial illumination. Hence the possibility of photosynthesis taking place after 10 to 12 minutes appears to be out of the question.

If then photosynthesis after 10 to 12 minutes is to be considered an impossibility the only explanation of the stepwise termination of the output of carbon dioxide during the first four light-dark periods in our experiments appears to be a gradual blocking of the mechanism of photo-oxidation. This blocking then must be caused by photochemical as well as by ordinary metabolic reactions. It is increased in the dark, but there is also a certain light effect, since long uninterrupted dark periods do not bring photo-oxidation to an end. Curves E and F in Figure 3 do not show any stronger reduction of the rate of photo-oxidation after a 40 minute dark period than do curves A and B after 10 to 11.5 minutes duration of the dark.

It is still a moot question which of the interpretations of the data here presented is the right one, whether photosynthesis occurs immediately after the first short light and dark treatments, or whether it is delayed and becomes a fact only after an uptake of carbon dioxide above the respiration level can be demonstrated, and whether in the latter case photo-oxidation is blocked by other reactions specific to this blokage. The experiments on which the present report is based have disclosed several previously unknown phases in the development of the photosynthetic mechanism in etiolated plants; but so far the data are too incomplete to allow one to draw any certain conclusions. More systematically planned studies are required in order to solve the problems effectively.

Summary

1. The carbon dioxide and oxygen exchange of intact etiolated wheat seedlings illuminated for the first time with protochlorophyll/chlorophyll converting light (white light 10.000 lux) is studied by means of the diaphragm method.
2. At continuous illumination a formation of carbon dioxide combined with a consumption of oxygen is observed immediately after the light is switched on. This gas exchange is considered to be due to a photo-oxidation process. At uniform illumination the said formation and consumption continue at an unchanged rate for up to 25 minutes (longest duration of individual experiments so far); a reduction of the light intensity causes a reduction of the gas exchange rate.
3. At intermittent illumination (light periods of 2 minutes, dark periods of from 10 to 11.5 minutes) the rate of photo-oxidation diminished from the first to the second, from the second to the third, and from the third to the fourth light period; during the fifth light period an uptake of carbon dioxide is observed; this is interpreted as the first infallible sign of photosynthesis (see point 5 below).

4. The duration from the onset of illumination until uptake of carbon dioxide is observed is found to be independent of the length of the light periods within the variations used (from 3 seconds to 2 minutes), but does to a certain extent depend upon the length of the dark periods. When alternating periods of 2 minutes light and 2 minutes dark are used it is possible to observe photosynthetic activity through uptake of carbon dioxide after 36 minutes. When 10 to 11.5 minutes dark periods are used it takes about 48 to 50 minutes before said observation is made.
5. It is discussed whether the stepwise reduction of the rate of photo-oxidation during the first light periods is due to an early, gradual starting of the photosynthetic process, or whether it is caused by a specific blockage mechanism which gradually inactivates the photo-oxidation reactions.

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Influence of Gibberellic Acid on Metabolism of Amino Acids

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Introduction

The stimulatory effect of gibberellin on germination of seeds (Wittwer and Bukovac 1957, Nekrasova 1960 and other numerous works), as well as changes in organogenesis, in the anatomic structure and in numerous physiological processes caused by application of gibberellin to seeds (Paskevič 1956, Herich 1960, Wittwer and Bukovac 1957, 1958, Nekrasova 1960 and others), induced us to study the influence of gibberellin on the metabolism of physiologically important substances in germinating seeds more precisely. In the following treatise are recorded some of our observations on the influence of gibberellin on the metabolism of free amino acids.

Material and Methods

Experimental study was carried through on seeds of hemp, var. "Ratislavická. Gibberellin was applied in concentrations of 5, 10, 25, 50, 100 and 200 ppm. Hemp seeds were placed in Petri dishes between two sheets of filter paper which had been moistened with the said gibberellin solutions, for control with distilled water. Germination took place in darkness, the temperature being 21°C.

For identification of amino acids the method of paper chromatography (descending) was used. We were working with a mixture of *n*-butanol, acetic acid and water 4 : 1 : 5. To get the greatest possible division effect we developed the chromatograms repeatedly eight times. Detection was carried out by 0.2 % ninhydrine solu-

tion in acetone. After detection the chromatograms were dried at a temperature of 60°C and 8 to 10 hrs. later photographed in penetrating light. Quantitative statement of amino acids was made from photographic negatives by microphotometric methods. The sensitivity of the photometer was adjusted in a way that all spots on the negative were measurable, shove of board being 0.2 mm. To eliminate the differences between the negatives caused by development or by staining the basis of the chromatograms, on each chromatogram the standards in the respective concentration were recorded for the purpose of drawing the calibration curves. The readings were made with technical help of Mr J. Navara.

The seeds were analyzed at the stages full swelling and beginning germination, length of germs up to 0.5 cm.

The following amino acids were rated: lysine, histidine, asparagine, aspartic acid, glycine, glutamine, alanylglycine, alanine, tyrosine, valine, methionine, leucine and phenylalanine.

Results and Discussion

Table 1 shows the results obtained on the influence of gibberellin on the process of metabolism of the various amino acids and amides during seeds germination.

From this table it is obvious that by application of gibberellin to seeds the metabolism of the observed amino acids and amides really was influenced in the course of germination. Changes took place both in quality and quantity. Of all amino acids observed only glycine, valine+methionine do not show essential changes after application of gibberellin during germination.

The observed changes in the metabolism of amino acids, due to application of gibberellin, point to a possibility of an active influence of gibberellin

Table 1. *Metabolism of free amino acids and amides during the germination of hemp seeds.* I — swelled seeds (not germinated), II — germinated seeds (length of germs up to 0.5 cm), quantity expressed in $\mu\text{g}/0.02$ ml. of extract; 10, 100, 200 ppm — concentrations of gibberellin.

	I.				II.			
	0	10 ppm	100 pmm	200 pmm	0	10 ppm	100 pmm	200 pmm
Lysine.....	0	0	0	0	5	0	0	0
Histidine	3	4.5	4	4.5	2	0	0	0
Asparagine.....	5	3	3	3.5	7	8	4	4
Aspartic acid	3	4	4	5	3	1	1	1
Glycine	1	1	1	1	2	2	1	1
Alanylglycine	1	2.5	4	5	24	24	4.5	5
Alanine	11	11	18	30	15	10	11	11
Glutamine		in traces				in traces		
Valine + Methionine	0	0	0	0	1	1	2	2
Leucine + Phenylalanine	0	1	2	4	3	2	3.5	3.5

on both the synthesis and desamination of amino acids during germination. Special attention in this respect deserves the increased content of alanine observed in swelled seeds after gibberellin was applied. The increase of the alanine content in a parallel with the gibberellin concentrations bears witness to close relation between gibberellin and the synthesis of alanine. At the supposition that at oxydative desamination of alanine in germinant seeds pyruvic acid is produced — a bridge connecting the metabolism of proteins, carbohydrates, and fats — it is probable that application of gibberellin finds its expression also in the metabolism of carbohydrates and fats as well as of oxydation and reduction processes which are closely related to the metabolism of proteins and amino acids.

For detailed study of the mechanism of gibberellin activity according to our opinion further complex research of the basic metabolic processes including amino acids is needed, this all the more because several growth substances are produced just as the result of the metabolism of amino acids by which the mechanism of gibberellin activity on the basis of auxins could be explained. Detailed study of the influence of gibberellins on the metabolism of the particular amino acids in relation to synthesis of growth substances will be the subject of our further work.

Samples of gibberellin have been obtained through the courtesy of Merck and Company, Rahway, New Jersey, Eli Lilly and Company, Indianapolis, Indiana, Kyowa Fermentation Industries, Tokyo, and from Plant Protection Limited, Farnham Research Station, Surrey, England.

Summary

The paper discusses the influence of gibberellic acid on metabolism of free amino acids and amides during germination of hemp (*Cannabis sativa*) seeds.

In the course of the germination quantity of the following free amino acids and amides were rated: Lysine, histidine, asparagine, aspartic acid, glycine, glutamine, alanyl-glycine, alanine, tyrosine, valine, methionine, leucine and phenylalanine.

In the spectrum of amino acids qualitative changes in the amounts of lysine, histidine, leucine rated together with phenylalanine, were induced by gibberellin treatment.

Quantitative changes were registered in the amounts of aspartic acid, asparagine, alanine, alanyl-glycine, lysine, leucine rated together with phenylalanine, glycine, and valine rated together with methionine.

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Effect of Red and Far-Red Irradiation on Nucleotide Phosphate and Adenosine Triphosphate Levels in Dark-Grown Bean and Avena Seedlings

By

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Red light (600—700 m μ) induces a number of morphological responses in plants (10, 15, 19) which can be inactivated by far-red (700—750 m μ) irradiation (3, 7, 10). Gordon and Surrey (4) have shown that oxidative phosphorylation by rat liver and Avena mitochondria can be affected by red and far-red irradiation. However, the major portion of the influence on oxidative phosphorylation in mitochondria appears to be in preserving the phosphorylating system rather than in stimulating it. Since growth responses require adenosine triphosphate (ATP) as a source of energy, a direct effect of light on the non-photosynthetic production of ATP could account for light-induced growth responses. If the growth response induced by red light is owing to an increase in high energy phosphate, the concentration of ATP in the tissue should increase upon irradiation with red light.

The purpose of this study is to investigate the ATP and nucleotide phosphate levels in various parts of the bean plant under conditions which would cause a photomorphogenic response and to evaluate the possibility that the red, far-red response is due to an alteration of the ATP or nucleotide phosphate levels in the tissue.

Materials and Methods

Biological material. Black Valentine bean plants (*Phaseolus vulgaris*) were grown in the dark as previously described (10). Plants were harvested using a photomorphogenic safelight (21) and were cut into sections. Intact leaves and three sec-

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tions of the hypocotyl were used; one section consisted of a segment approximately 1 cm. in length of the curved portion (hook) immediately below the cotyledons, another consisted of a 1 cm. segment taken from the expanding region below the hook section, and the third consisted of the remainder of the hypocotyl. Whole plants and plant parts were exposed to red (625—700 m μ) or far-red (725—1150 m μ), using filters (20) to obtain the desired wavelengths.

Avena were grown on porous silica wicks (18) or on vermiculite in a saturated atmosphere and were harvested 85—90 hours after the start of water imbibition.

Nucleotide phosphate. Plant material was ground by hand in a chilled mortar with cold 10 % perchloric acid and extracted twice with 5 % perchloric acid. After removing insoluble material by centrifugation, nucleotide phosphate was adsorbed on activated charcoal, as described by Marré and Forti (13). After the charcoal was washed with water to remove non-nucleotide phosphate, it was suspended in 1 *N* HCl and placed in boiling water for 10 minutes. The charcoal was removed by centrifugation and the phosphate determined by the method of Bernhart and Wreath (2).

In experiments involving P³², the charcoal was collected on a fritted glass filter and, after washing five times with the water, the nucleotide phosphate was eluted by washing three times with 50 % ethanol — 1 % NH₄OH (8). The eluate was evaporated to dryness, and P³² determined by use of a geiger counter.

Total organic phosphate. Organic phosphate was separated from inorganic phosphate by the method of Marsh (14), and the activity in the organic and inorganic fractions was determined with a geiger counter.

Adenosine triphosphate. Plant material was extracted as described for nucleotide phosphate. The perchloric acid was neutralized with KOH and the potassium perchlorate removed by centrifugation. This extract was diluted at least fivefold before assaying. Placing tissue in boiling water for 1 minute followed by blending in a high speed blender yielded amounts of ATP comparable to the perchloric acid extraction. This method was used to extract small amounts of tissue.

The amount of ATP was assayed by a luciferin-luciferase method of McElroy (personal communication), using a commercial source of firefly tails (Schwartz Bioresearch, Inc., 230 Washington St., Mt. Vernon, N.Y.), and removing transphosphorylases (1) from the firefly enzyme according to the procedure of Green and McElroy (5). The purification procedure was carried through elution from phosphate gel. This preparation produced only a very small response when a mixture of uridine triphosphate (UTP) (10^{-3} *M*) and adenosine diphosphate (ADP) (10^{-3} *M*) were tested as substrates.

The assay was made by injecting approximately 10^{-4} micromoles of ATP into the reaction chamber and measuring the light emitted on a specially built recording instrument. The reaction mixture contained 2.1 ml. glycyl-glycine buffer 0.025 *M*, pH 7.5, 0.1 ml. MgSO₄, 0.1 *M*, 0.1 ml. enzyme, and 0.5 ml. of sample. ATP (99 % pure) was used to calibrate the instrument.

Protein determination. Protein was determined by the method of Lowry, *et al.* (12). The intact tissue was extracted with boiling ethanol to remove soluble phenols and then homogenized in a Tenbroeck glass homogenizer. Bovine serum was used as a standard.

Chlorophyll determination. Chlorophyll was determined by a procedure previously described (11, 22).

Hook opening. Hook opening was determined by a procedure previously described (10).

Results

The distribution of 10-minute labile nucleotide phosphate in various parts of 6-day-old bean plants is shown in Table 1.

The amount of nucleotide phosphate on a fresh weight basis is greatest in leaves and higher in the hooks than in the roots and expanded stem. The area just below the hook is the area of most rapid elongation. This area also is lower in nucleotide phosphate than the non-expanding hook on a fresh weight basis. On a protein basis, all portions of the plant analyzed have approximately the same amount of nucleotide phosphate.

The distribution of ATP in various parts of the bean plant is shown in Table 2. The general pattern of distribution is the same as for 10-minute labile nucleotide phosphate. On a fresh weight basis, the leaves have more ATP than the hook. The hook has more ATP than elongated hypocotyl. The inner portion of the hook has approximately 30 % more ATP than the outer portion. On a protein basis, all parts of the plant tested have about the same amount of ATP.

In order to determine if a direct analysis of tissue for ATP would be a valid estimate of the ATP available for growth, the effects of DNP (2,4-dinitrophenol) on ATP levels and growth responses were determined. Dinitro-

Table 1. Nucleotide levels in 6-day-old Black Valentine beans grown in darkness.

Part	10-minute labile nucleotide phosphate ¹	
	$\mu\text{mol./g. fresh weight}$	$\mu\text{mol./mg. protein}$
Leaf	0.95	0.016
Hook	0.57	0.017
Segment below hook...	0.29	0.016
Elongated segment ...	0.09	0.015

¹ Phosphate adsorbed on activated charcoal and liberated by 1 N HCl at 100°C for 10 minutes. Average values for 3 experiments.

Table 2. Summary of ATP levels in 6-day-old Black Valentine beans grown in darkness. Average values for 3 experiments.

Part	$\mu\text{mol./g. fresh weight}$	$\mu\text{mol./mg. protein}$
Leaf	0.32	0.0041
Hook	0.14	0.0040
Inner portion of hook	0.16	0.0040
Outer portion of hook	0.12	0.0040
Segment below hook..	0.07	0.0040
Elongated segment ...	0.025	0.0042
Roots	0.04	0.0040

Table 3. *Effect of dinitrophenol on ATP level and growth processes in 6-day-old dark-grown bean plants.* Values denote per cent of control level per unit fresh weight. Time of treatment for ATP level and chlorophyll synthesis — 18 hours. Time of treatment for hook opening — 20 hours. The control leaf synthesized 100 μg of chlorophyll/g fresh weight and the control hook synthesized 6.7 μg of chlorophyll/g fresh weight. No significant hook opening occurred in the dark.

DNP concentration (M)	ATP in leaves	ATP in hooks	Chlorophyll synthesized in leaves	Chlorophyll synthesized in hooks	Hook opening (light)
0	100	100	100	100	100
10^{-5}	90	90	90	85	—
5×10^{-5}	70	70	66	68	72
10^{-4}	50	45	47	48	58

phenol is a known uncoupler of oxidative phosphorylation. The effect of DNP on the ATP levels of leaf and hook is shown in Table 3. The concentration of DNP necessary to decrease ATP was somewhat lower than that found by Syrett in *Chlorella* (16), but conditions were considerably different. Similarly, the effect of DNP on chlorophyll synthesis is shown. The percentage of inhibition correlates very well with the percentage of lowering of ATP in these tissues. It is assumed here that a process as complex as chlorophyll synthesis would require ATP. Hook opening is inhibited by DNP, and these values are in close agreement with the trend shown by chlorophyll synthesis and the ATP concentration.

Effect of red light on ATP and nucleotide phosphate levels. In order to determine if red light would alter the ATP level in the bean hook, intact plants were irradiated with 100 $\mu\text{w}/\text{cm}^2$ of red (625—700 m μ). At various time intervals, hooks were harvested, weighed, and the ATP extracted. Similarly, hooks were harvested from plants kept in darkness. Very little difference in ATP level was detected between plants irradiated with red light and plants kept in darkness (Table 4). In a number of experiments, the

Table 4. *Effect of 100 $\mu\text{w}/\text{cm}^2$ of red light on ATP levels in hooks of dark-grown Black Valentine beans.* No change in protein content could be detected during the treatment time. Average values for 3 experiments.

Time (hr)	$\mu\text{mol./g. fresh weight}$	
	Light	Dark
1	0.14	0.14
3	0.13	0.15
6	0.13	0.14
20	0.13	0.14

Table 5. *Effect of 100 $\mu\text{w}/\text{cm}^2$ of red light on nucleotide phosphate levels in dark-grown Black Valentine bean hook. Phosphate adsorbed on activated charcoal and liberated by 1 N HCl at 100°C in 10 minutes. Average values for 2 experiments.*

Time (hr)	10-minute labile nucleotide phosphate			
	$\mu\text{mol./g. fresh weight}$		$\mu\text{mol./mg. protein}$	
	Dark	Light	Dark	Light
1	0.60	0.60	0.018	0.018
3	0.60	0.61	0.018	0.018
6	0.60	0.61	0.018	0.018

ATP level appeared to be slightly lower in hooks irradiated by red light. However, in the red-irradiated plants, chlorophyll synthesis, utilizing ATP, was taking place in addition to the photomorphogenic reactions, thus probably accounting for the lower ATP level. The difference in amount of ATP was not pronounced and could be considered within the limits of experimental error. At the end of the experiment, the hooks kept in the light were nearly open.

Similarly, a comparison was made between the ATP levels of the inner and outer portions of the hook! Again, no significant difference in ATP level with time could be shown upon irradiation with red light. No effect of red light on 10-minute labile nucleotide phosphate could be detected (Table 5).

Effect of far-red irradiation on ATP level. Plants were irradiated with far-red (725—1150 $\text{m}\mu$) and the ATP level in the hook determined at various time intervals (Table 6). No difference in ATP level could be detected during the time interval.

Effect of light on ATP level in Avena coleoptile and mesocotyl. Further experiments to determine if the ATP level is influenced by light were attempted using Avena coleoptile and mesocotyl. Red light stimulates the growth of the Avena coleoptile and inhibits the mesocotyl in a matter of a few hours (17). Light causes a decrease in ATP on a fresh weight basis (Table 7) in the Avena coleoptile. However, on a protein basis, this decrease

Table 6. *Effect of 100 $\mu\text{w}/\text{cm}^2$ of far-red light on ATP levels in hooks of dark-grown Black Valentine beans. No change in protein content could be detected during the treatment time. Average values for 2 experiments.*

Time (hr)	$\mu\text{mol./g. fresh weight}$	
	Light	Dark
1	0.13	0.13
3	0.14	0.14
6	0.14	0.14

Table 7. *Effect of 30 $\mu\text{w}/\text{cm}^2$ of red light on ATP levels in dark-grown Avena coleoptile and mesocotyl. Average values of 3 experiments.*

Light treatment (hr)	Coleoptile		Mesocotyl	
	$\mu\text{mol./g. fresh weight}$	$\mu\text{mol./mg. protein}$	$\mu\text{mol./g. fresh weight}$	$\mu\text{mol./mg. protein}$
0	0.121	0.0054	0.062	0.0061
1	0.120	0.0055	0.062	0.0060
3	0.104	0.0046	0.060	0.0060
8	0.090	0.0050	0.062	0.0057

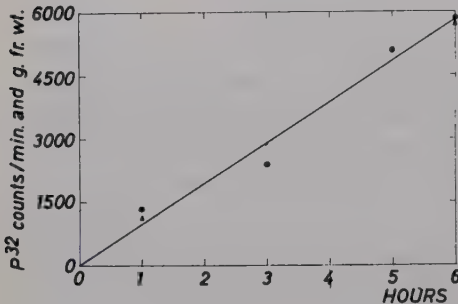


Figure 1. *Incorporation of P^{32}O_4 into nucleotides of the inner portion of Black Valentine bean hooks in 30 m μ watts of red light (600—700 m μ) and in darkness. The tissue was incubated with P^{32}O_4 (approximately 1 $\mu\text{c}/\text{ml}$) for the indicated time.*

▲=light; ●=dark.

is not significant. In the mesocotyl, the ATP level is unaltered during the time interval studied.

Incorporation of P^{32}O_4 into organic phosphate. Attempts were made to determine if light would increase the incorporation of P^{32}O_4 into organic phosphate of bean hooks. A number of experiments failed to reveal any increase of incorporation of P^{32}O_4 into total organic phosphate. Likewise, light failed to stimulate the incorporation of P^{32}O_4 into nucleotide phosphate (Figure 1). Experiments in which light was applied 8 hours before the start of the experiments did not reveal an increase in the P^{32} -labelled nucleotide phosphate during 6 additional hours.

No difference could be observed in the rate of incorporation of P^{32} into nucleotides of *Avena coleoptiles* in red light or darkness during a 6-hour treatment. Determinations were made during the treatment period at 0, 1, 3, and 6 hours.

Discussion

The distribution of nucleotide phosphate and ATP in various portions of the dark-grown Black Valentine bean plant is approximately proportional to the protein content of the tissue (Table 1), although the variation on a fresh weight basis may be quite large.

On a protein basis, the amount of ATP in tissues of the Black Valentine bean which are undergoing rapid expansion (segment below hook) is about equal to that in tissues which are not undergoing expansion (inner portion of hook), but which later expand (Table 2) in darkness. If the amount of ATP present in tissues is a valid measure of the amount of ATP available for growth, this would indicate that ATP is not the growth-limiting factor in the tissues which are not expanding.

Red light will induce the tissue on the inner portion of the hook to expand, and some evidence in the literature suggests the possibility that light influences the amount of ATP available. The interesting experiments of Gordon and Surrey (4) indicate that oxidative phosphorylation merits serious consideration as a biochemical basis for the red, far-red reaction. In addition, Haber (6) has shown that light-insensitive lettuce seed may be made light-sensitive by dinitrophenol. When irradiated with either red or far-red, bean hook tissues display no substantial change in the amount of ATP. It is possible that light can alter the amount of ATP to some extent. However, if this is true, the change must be too small to be detected by the methods used in this study.

In the light, dinitrophenol (10^{-4} M) lowers the ATP content of bean hooks 55 %; however, the hook opening is inhibited 42 % (Table 3). These experiments would suggest that hook opening is dependent on ATP, but that ATP is not the limiting factor in the light-induced hook opening. The dark control had no significant opening during this period and thus supports the view that ATP is not the limiting factor in expansion, since the ATP concentration is approximately twice as great as in the dinitrophenol (10^{-4} M) treated hook.

It is possible that light could increase the rate of ATP formation and utilization with only a transient rise in the ATP level. Attempts were made to determine this possibility using $P^{32}O_4$. No difference in the rate of incorporation of P^{32} into organic phosphate or nucleotide phosphate was detected. Such an experiment is complicated by the fact that incorporation of $P^{32}O_4$ into organic phosphate involves an accumulative process in addition to a synthetic process. The time necessary to accumulate phosphate (9) might be relatively long as compared to the incorporation time. The fact that no differences were observed does not necessarily prove that the rates of formation and utilization of organic phosphate compounds were the same in light and in darkness.

These experimental results indicate that red and far-red radiant energy apparently do not regulate growth responses by altering the available

amounts of ATP. It would seem more likely that if the high energy phosphate system is involved, the radiant energy effect is the redirection or channeling of the ATP into a specific utilization process.

Summary

The adenosine triphosphate (ATP) content of Black Valentine bean seedlings and of the *Avena* coleoptile and mesocotyl has been investigated in relation to red (600—700 m μ) and far-red (700—750 m μ) radiant energy responses. The nucleotide phosphate and ATP contents of the leaves, hypocotyl hook, elongated portion of the hypocotyl, and roots of 6-day-old dark-grown bean plants were proportional to the protein content. No detectable differences in the ATP level could be measured upon irradiation of the bean hooks with either red or far-red radiant energy. Dinitrophenol (10^{-4} M) reduced the ATP level of hooks to approximately 50 % of the level of control hooks. When exposed to red radiant energy, the dinitrophenol-treated hooks of bean plants responded to about 60 % of the response induced in the control. The ATP levels in the coleoptile and mesocotyl of *Avena* also were not influenced by red light. These results are interpreted as indicating that ATP is required for a light-induced growth response to occur, but alteration of the ATP level by red, far-red irradiation is probably not the system regulated by the photoreaction.

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Ion Absorption in Young Sunflower Plants II. The Sulphate Uptake in the Apparent Free Space

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In an earlier paper (Pettersson 1960) the general trends of the sulphate uptake to intact sunflower plants were presented. In this communication some experiments have been collected where the initial phase of the sulphate uptake in the sunflower roots has been given special attention. Thus attempts have been made to study the time course of the initial uptake more in detail than is generally done in AFS-determinations. Further the magnitude of the AFS of attached and excised roots has been compared at different medium concentrations. This question has been under some discussion during the last few years (Kylin and Hylmö 1957, Jacobson *et al.* 1958). The active uptake as influenced by the ion concentration in the free space was recently discussed by Bernstein and Nieman (1960). Some new results concerning this question are also presented here. Finally the observed influence of some inhibitor substances on the initial uptake has been given some attention.

Materials and Methods

Sunflower plants, *Helianthus annuus*, var. Californicus, were grown for 12 days in water culture at 25°C and constant illumination of an intensity of approximate 9000 lux. The composition of the standard nutrient solution was

2 mM KNO₃; 1 mM Ca(NO₃)₂; 0.5 mM MgSO₄; 1 mM KH₂PO₄; 0.5 mM Na₂HPO₄; 10 mg/l ferricitrate; 0.1 mg/l MnSO₄; 0.1 mg/l H₃BO₃.

In concentration series under Experiments section b) the following media were used in the experiments

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0.25 mM SO ₄ :	standard nutrient solution with 0.25 mM MgSO ₄ ;
0.5 mM SO ₄ :	" " " ;
5 mM SO ₄ :	" " " plus 1 mM Na ₂ SO ₄ , 3.5 mM K ₂ SO ₄ ;
50 mM SO ₄ :	" " " " 12 mM Na ₂ SO ₄ , 37.5 mM K ₂ SO ₄ .

Experiments under section b) were carried out in the photothermostats where the plants had been raised. In all other cases the experiments were performed in a laboratory with the air temperature $21^{\circ} \pm 1^{\circ}\text{C}$. The plants were transferred to this room 15 hours before the experiment proper. Other environmental factors were unchanged during nursery, pretreatment and experimental periods.

The nutrient solutions were labelled with a suitable amount of radioactive sulphate during the experiments.

The volume of the test solutions was 800 ml. for 2—4 g. roots or for intact plant groups weighing about 15 g. and no changes in medium concentrations during the experiments could be detected. Aeration of all solutions was arranged for. Excision of roots was performed 30 minutes before the experiments.

After the experimental periods the roots were blotted between filter papers according to the standardized method.

The radioactivity was measured by GM-counting on BaSO₄-precipitates of standard area and weight.

For a more detailed description of culture conditions and analytical methods see Pettersson (1960).

Experimental Results and Discussion

a. Time course of the initial uptake

In the AFS-determinations which have been carried out during the last years the experimental times have generally been rather long. Especially for the uptake of cations times of more than one hour have been regarded necessary for full equilibration. Thus Briggs *et al.* (1958) reported an equilibration time in the AFS for Rb of 90—100 minutes and for Ca of 200 minutes when using beet root discs at 2°C . For Br and I the somewhat shorter time 20—30 minutes was reported. Brouwer's (1959) excised pea roots were "fully equilibrated" after 30—60 minutes with regard to Rb and Butler (1959) speaks of "the initial 30 minutes". Jacobson *et al.* (1958) working with excised barley roots, writes that nonmetabolic uptake occurred at least 60 minutes for K but for HPO₄⁻ "equilibrium had been reached within 5 minutes". Briggs (1957, Figure 1) indicates that the AFS uptake of KCl would be completed within about 8 minutes. Kylin and Hylmö (1957) worked with wheat. The AFS of their excised roots was filled in 15 minutes and at least 70 per cent of the equilibration was concluded during the first minute. Working with isolated walls of *Chara australis* Dainty and Hope (1959) obtained the extremely short exchange times of about 1 second for the equilibration between medium and AFS. Bernstein and Nieman (1960) used equilibration times from 15 minutes and longer with different mate-

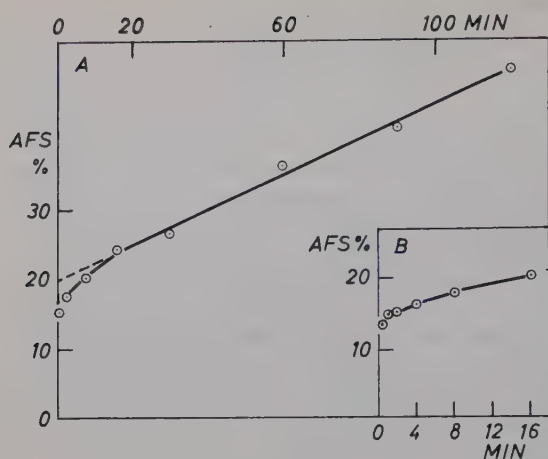


Figure 1. Initial sulphate uptake in excised sunflower roots. Standard nutrient solution with 0.5 mM sulphate. Temperature $21^{\circ} \pm 1^{\circ}\text{C}$. Vertical axis gives the ion uptake calculated as per cent AFS. Horizontal axis represents the time in labelled solution. The upper time scale refers to Figure 1 A, the lower scale to Figure 1 B. Dotted line — AFS-extrapolation. Shortest experimental time in Figures 1 A and 1 B 30 seconds. Each point represents the mean of two determinations on different samples.

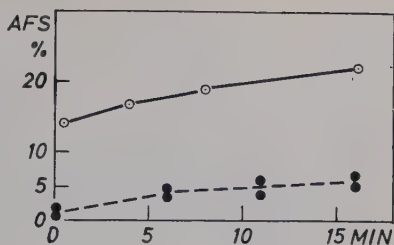
rials. In observations and in a theoretical discussion these authors show that for bean roots 78 per cent equilibration was reached within 15 seconds and after 5 minutes more than 90 per cent of the ions in the AFS were exchanged for medium ions.

In the following some experiments will be described which are intended to give some elucidation to the velocities of the ion exchanges which are going on between the medium and the free space in experiments with sulphate.

In Figure 1 the time course for the uptake of sulphate to excised sunflower roots is represented. Figure 1 A gives a survey of the uptake over 2 hours, showing the constant rate of accumulation after the AFS equilibration and the AFS extrapolation. Figure 1 B represents in detail the rapid filling of the AFS. The first sample is taken after 30 sec. absorption time and as much as 70 per cent of the AFS seems to be already filled here.

In Figure 2 the AFS of blotted roots is compared with that of roots which have been rinsed in distilled water for 10 seconds before blotting. A decrease of AFS corresponding to 13–15 per cent of the root volume is obtained at every length of the preceding absorption period. As the total AFS according to Figure 1 is about 20 per cent a considerable fraction of this amount is thus lost during the first 10 seconds of rinsing. These values are quite in accordance with those obtained by Brouwer (1959) for Rb. Thus Brouwer reported an initial uptake of 80 μg . Rb/10 mg. dry wt. root after blotting the roots, whereas the corresponding quantity after rinsing 10–20 seconds in distilled water reached only 15 μg . Rb. Levitt (1957) and recently Ingelsten and Hylmö (1961) have theoretically and experimentally showed that a considerable part of the AFS values obtained by blotting methods refers

Figure 2. Effect of rinsing sunflower roots with distilled water before determination of the AFS for sulphate. Standard nutrient solution with 0.5 mM sulphate. Temperature $21^{\circ} \pm 1^{\circ}\text{C}$. Vertical axis: ion uptake calculated as percent AFS. Horizontal axis: time in labelled solution. Open symbols — values from Figure 1 A. Filled symbols — values obtained after rinsing root bundles 10 seconds in distilled water immediately after absorption period.



to a contamination of medium adhering to the surface of the roots. Even though the inner limit of such a liquid film seems to be somewhat difficult to define the correction may be applied. The "inner" AFS of these sunflower roots at 0.5 mM SO_4 would thus amount to 10–12 per cent after removing the surface contamination, which would contribute to about 8–10 units of the AFS-value. In the light of these figures the considerable loss of ions from the rinsed roots of Figure 2 is not too surprising, as only about 40 per cent of the "inner" AFS would have been emptied by the rinsing. The values of Figure 2 are in rather good accordance with those presented by Bernstein and Nieman (1960) for exodiffusion of mannitol and chloride.

b. AFS in excised roots and in roots of intact plants

Due to the widely different results obtained by other investigators (Kylin and Hylmö 1957; Jacobson *et al.* 1958) regarding the effect of excision of roots on the computed values of the AFS the following experiments were performed.

The general arrangement of the experiments was as follows. Four root bundles or two intact plant groups on their holders were put into radioactive test solution simultaneously. After 40 minutes one of the root bundles was taken for analysis after blotting the roots, and another after 160 minutes. The remaining two bundles of excised roots were transferred to inactive solution after 160 minutes. After 40 and 160 minutes respectively in this solution these roots were taken for analysis. The AFS was then obtained by extrapolation of the uptake and leakage curves. Leakage of radioactive roots in distilled water has given the same rate of the sulphate outflow as when inactive complete nutrient solution was used. In these experiments inactive complete nutrient solution has been used for leakage experiments.

The results are presented in Figures 3 and 4. For intact plants the AFS of the roots is nearly constant at 50, 5, and 0.5 mM sulphate (Figure 3) but at 0.05 mM adsorption of sulphate to plasma or wall constituents seem to have some influence on the measurements (cf. Epstein 1955 and Butler

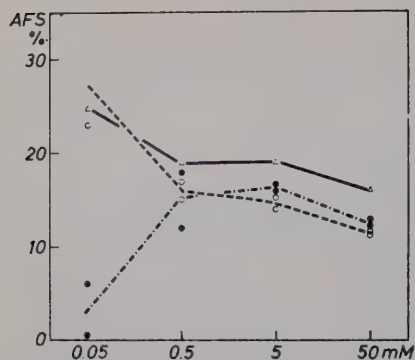


Figure 3. AFS for sulphate in attached and excised sunflower roots at different sulphate concentrations in the medium. Temperature 25°C. On the horizontal axis sulphate concentration of the test solution. — *Triangles*: AFS in attached roots obtained by extrapolation of accumulation curve according to Figure 1. — *Open circles*: AFS in excised roots obtained by extrapolation of accumulation curve according to Figure 1. — *Filled circles*: AFS in excised roots obtained by extrapolation of leakage curve, when leaching in inactive nutrient solution. — For attached roots every point is the mean of 6 determinations, for excised roots each point is the mean of 2 determinations.

1959). For excised roots the value of AFS is as high as that for roots of intact plants at 0.05 mM sulphate, but at the higher concentrations the values obtained are 3–5 units lower. The AFS computed after leaching the roots is presented, too, in Figure 3. The values obtained are very small in this case at 0.05 mM sulphate, while at the other concentrations no significant differences from the absorption experiments could be detected. In Figure 4 the accumulated sulphate at the time interval from 0.40 to 2.40 hours is represented. At all investigated concentrations of the ambient solution the accumulation by roots of intact transpiring plants is higher than the corresponding amount by excised roots.

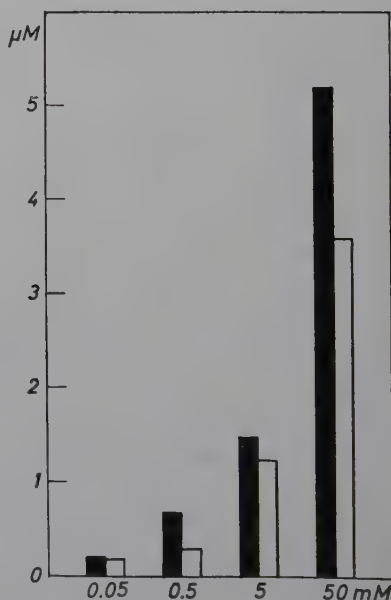


Figure 4. Active sulphate retention in attached and excised sunflower roots at different sulphate concentrations in the medium. Temperature 25°C. Filled columns: Retention in attached roots. Empty columns: Retention in excised roots. Filled columns means of 6 determinations; empty columns means of 4 determinations. All values refer to root fresh weight 4 g.

In experiments with wheat Kylin and Hylmö (1957) reported a mean AFS of 18 per cent for excised roots and a value of 27.5 per cent for intact roots when using sulphate concentrations 0.05, 0.5, and 5 mM. Using 20.5 mM phosphate Jacobson *et al.* (1958) computed a value of AFS of 31.5 per cent in excised roots and of 20.5 per cent in intact roots in their experiments with barley. Disregarding the widely differing volumes of the AFS indicated in these investigations, which may be due to different materials and experimental methods, the general trend in the differences between the AFS values of intact and excised roots are quite opposite. In the present investigation *no support can be given to the values reported by Jacobson et al. (1958), while at concentrations above 0.5 mM sulphate the trends reported by Kylin and Hylmö (1958) have been confirmed.* Bernstein and Nieman (1960) report trends to higher AFS values in roots of rapidly transpiring plants than in slowly transpiring ones due to concentrating of the AFS solution, as water is rapidly removed by ultrafiltration and transported to the shoots of the transpiring plants.

The data of Figures 3 and 4 seem to be well explained by the above hypothesis. The AFS is greater in roots of intact transpiring plants than in excised roots with no transpiration stream. Leaching of the excised roots shows an exodiffusion of the same magnitude as that of the endodiffusion. That means that no dilution of the AFS solution has occurred in these roots. Then, if the free space volume is assumed to be the same for excised and attached roots, there must be a concentration of the solution in the free space of the attached roots as the amount of sulphate in the free space is greater. The data of Figure 4 lend support to this view. The active uptake in the attached roots is of a greater magnitude than that of the excised roots at all concentrations. Such an increased active uptake may, for the same ion at a given temperature, be due to either an increased absorption surface or to an increased concentration of the solution limiting the absorbing surface. As the latter alternative is the more plausible in this case the consequence will be an increased ion concentration in the AFS of the attached roots.

c. The influence on the AFS by inhibitor substances

In some experiments the influence of sodium-selenate and 2,4-dinitrophenol on the initial sulphate uptake has been given attention. The results are presented in Figures 5 A and 5 B.

The experimental procedure was the same as described for the previous sections. The inhibitor substances were added to the experimental solutions only. That means no action by the inhibitor was allowed before the uptake experiment proper. As can be seen from Figure 5 A the active uptake was

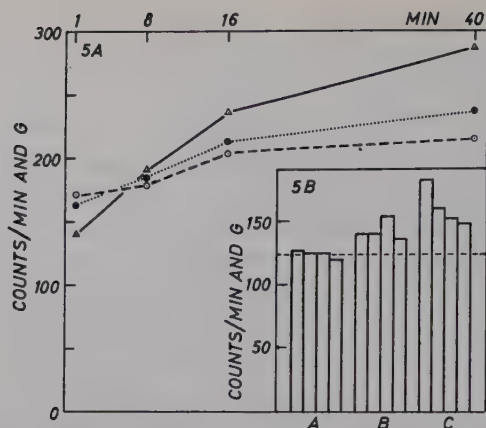


Figure 5 A. Time course of the sulphate uptake in excised sunflower roots when inhibitors are added to the medium. Standard nutrient solution with 0.5 mM sulphate. Temperature $21^{\circ} \pm 1^{\circ}\text{C}$.

Triangles — in standard nutrient solution.

Open circles — " " " " + 10^{-4} M 2-4-DNP + 20 mM Na_2SeO_4 .

Filled circles — " " " " + 10^{-4} M 2-4-DNP.

Each symbol represents the mean of 2 determinations.

1 ml nutrient solution corresponds to 1340 counts per minute.

Figure 5 B. Sulphate uptake in excised sunflower roots during the first minute of absorption when inhibitors are added to the medium. Medium and temperature as in Figure 5 A.

A — in standard nutrient solution.

B — " " " " + 10^{-4} M 2-4-DNP and 20 mM Na_2SeO_4 .

C — " " " " + 20 mM Na_2SeO_4 .

Each column represents one single determination.

1 ml nutrient solution corresponds to 1340 counts per minute.

considerably inhibited by sodium selenate and 2-4-DNP. No visible damages from the inhibitor treatments on the root tissue could be detected during or after the experiments. As can be seen from Figures 5 A and 5 B the sulphate uptake by excised sunflower roots is significantly increased during the first minute of absorption if inhibitors like sodium selenate or 2-4-DNP are added to the nutrient solution.

An effect like this is not earlier described in the literature. It may be noted that what is increased is not the apparent volume of the free space but the rate of filling the AFS. If extrapolations from the uptake between 16 and 40 minutes were made in Figure 5 A an AFS of about 15 per cent would be

Table 1. AFS in roots of transpiring sunflower plants different sodium selenate levels in the experimental solution. Standard nutrient solution with 0.5 mM sulphate. Experimental temperature 25°C. AFS obtained by time differentiation between 30 and 150 minutes and extrapolation according to Figure 1. All figures are means of 4 determinations with maximal deviations from means indicated.

Selenate conc. in nutrient solution (mM)	A F S %	Maximal deviation units
0	18	1
0.5	25	4
1	22	2
20	17	2

obtained from all curves. Thus the effect cannot be interpreted as a result of any additional cellular region being available for diffusion. Instead there seems to be some kind of process facilitating the filling of the free space. If this volume is understood as a pore system with charged walls (cf. Hylmö 1958, Dainty and Hope 1959) some change in pore size which should be equivalent to an increase of the general permeability may give the effect. Further experiments are required to elucidate these phenomena.

In this connection a source of error in estimating the volume of AFS when using inhibitors may be mentioned. Table 1 gives the AFS values obtained by time differentiation at different levels of sodium selenate present in the experimental solution. The experiment is carried out with intact plants and thus there may have been those concentration effects in free space which are described in section b. But as the transpiration is kept fairly constant in the different series, this effect may be negligible in comparison with the inhibitor effects.

It can be seen that at fairly low selenate concentrations there is a considerable increase in the AFS volume but at the same time the figures are more uncertain.

This effect may be ascribed to the time factor in the inhibiting action of the selenate. At moderate inhibitor concentrations the active processes adjust their rate to a lower and constant rate only after the time elapse of some hours. The rectilinear AFS extrapolations are, therefore, in these cases made from, in reality, convex lines and high values of the AFS will be the result.

The time effect described here is of importance not only in AFS estimations, but it must be taken into consideration also in computations of active uptake in short time experiments when inhibitors are used.

Summary

The initial rapid uptake (the uptake in AFS) of sulphate in roots of 12-day-old sunflower plants has been under investigation.

1. The time course of the initial uptake indicated a very rapid filling of the AFS. As much as 70 per cent of the free space seems to be filled during the first 30 seconds of the absorption period.

2. Roots of intact transpiring plants have a trend to higher AFS-values than those obtained for excised roots. The attached roots have also a more rapid active uptake than the excised ones. These effects may be due to an increased sulphate concentration in the AFS at rapid transpiration.

3. The rate of the uptake by excised roots was increased during the first minute of absorption if inhibitors like sodium selenate or 2-4-DNP were added to the nutrient solution. The effect is supposed to be due to some morphological change of the AFS or to some alteration of the general permeability caused by the inhibitor.

The author is indebted to Prof. H. Burström, Dr. B. Hylmö and B. Ingelsten for valuable discussions and to Mrs. M. Pettersson for skillful analytical work. The work has been supported by a grant from the Swedish Natural Science Research Council.

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Ultraviolet Action Spectra of Positive and Negative Phototaxis in *Platymonas subcordiformis*

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Introduction

Several action spectra analyses of phototaxis have recently been performed in the visible region for a number of different organisms (Bünning and Schneiderhöhn 1956, and Gössel 1957 on *Euglena gracilis*; Halldal 1958 on unicellular green algae, dinoflagellates and *Ulva*-gametes). These action spectra analyses reveal that topo-phototactic orientation in flagellates is generally restricted to the violet and green region of the spectrum with maximum sensitivity around 475 and 500 m μ . A few exceptions to this general rule are known. Negatively reacting *Euglena viridis* and also chlorofyll-free and colourless forms of this species have a maximum at 410 m μ and the dino-flagellate *Prorocentrum micans* has an action spectrum maximum at 570 m μ . The interpretation of these different action spectra is dealt with below under discussion.

The analyses performed in the visible region have yielded very few details in the action spectra, and it has not been possible without introducing speculation to draw conclusions about the photoreceptive pigment, or pigment type, that may be involved in the reaction. One may assume that these could be carotenoids, riboflavin or pterins, all with rather similar absorption characteristics in the visible region. These pigments do, however, have distinct absorption differences in ultraviolet. With this in mind action spectra analyses of topo-phototaxis were therefore extended to this spectral region.

Recently ultraviolet action spectra analyses of phototropism and growth have been performed with success in *Phycomyces sporangiospores* by Delbrück and Shropshire (1960) and Curry and Gruen (1959).

Materials and Methods

The unicellular brine Volvocales *Platymonas subcordiformis* (Wille) Hazen (Gibor's strain) was used. The algae were grown in a synthetic medium which was developed to meet requirements both for growth and phototactic response (Halldal 1960). In producing negatively or positively reacting cells the same procedure was used as earlier (*l.c.*). The action spectrum measurements implied threshold value determinations at different wavelengths. These were performed in the following way: 0.3 ml. of a sample was transferred to a 1 cm. path crystal quartz cuvette and the sample exposed to monochromatic radiation from the side for two minutes. The algal distribution was then checked with a horizontal microscope with dim red light as microscope illumination. In this way the lowest radiation which caused a distribution gradient of organisms was recorded. In order to avoid light-induced phototactic response changes the stock population was kept in the dark and a new sample was taken after every two minutes of radiation. The energy was measured, calculations were performed for incident number of quanta, and the reciprocal of this value plotted against wavelengths of radiation gave the action spectrum curve. A reference wavelength was selected at 405 m μ and the material was tested for the threshold response for this radiation at the start and at the end of each day and in between at usually one and a half hour intervals. During the whole experimental period the threshold value of phototactic response was exceedingly constant and it was therefore possible to give the ordinate of Figure 1 in absolute values.

Apparatus. A Bausch & Lomb 250 mm. grating monochromator with 600 grooves/mm. was used as source for the monochromatic radiation. The spectral band width was set at 5 m μ . Below 405 m μ the mercury lamp that comes with the monochromator was used, and from 380 m μ and up the incandescent lamp. In addition to the mercury lines spectral regions in between the lines were also isolated for irradiation. The quartz cuvette was placed for irradiation immediately outside the exit slit at the image of the grating. Radiant energy was adjusted by means of the Vee-slide of the exit slit which controls slit length. Adjustments at this place in the optical system change the intensity at the image of the grating uniformly. This could be controlled by looking at the image in the visible region. This principle of adjustment was further checked by taking measurements of phototactic threshold values at strong mercury lines, for example at 302, 313 and 365 m μ (nearly closed Vee-slide slit); and immediately outside the lines (open slit). No irregularities which could be attributed to measurements taken on the mercury lines or in between them could be observed (see Figure 1).

The energy was measured with a 1P 28 RCA photomultiplier tube. As it was not possible at this institute to make a sensitivity calibration curve for this particular tube, the relative spectral sensitivity curve given by the manufacturer had to be used. As production variations in the spectral response curve of this tube are small (Engstrom 1947) the procedure employed should be safe for our purpose. Based

upon this assumption the photomultiplier tube was calibrated in absolute values by energy measurements from the strong mercury line at 365 m μ isolated by the Bausch & Lomb monochromator. The radiant energy at this wavelength was measured by a Moll & Burger Standard Thermopile (E5), firm Kipp & Zonen in combination with a Kipp & Zonen portable galvanometer (Pa A, 70) which was calibrated for voltage deflection.

Results

The action spectra for positive and negative phototaxis are given in Figure 1. In order to complete the curves measurements were also performed in the visible region. In this part of the spectrum the curves give the general shape as in earlier investigations (Halldal 1958). A new detail was observed at 405 m μ where the action spectra have a small maximum. Halldal's results from 1958 are uncertain in this spectral region. In these measurements the response was tested against a reference beam. In applying this method the base of the action spectrum curves will be eliminated. In addition the energy of the incandescent lamp is low in violet. This will make readings of photo-

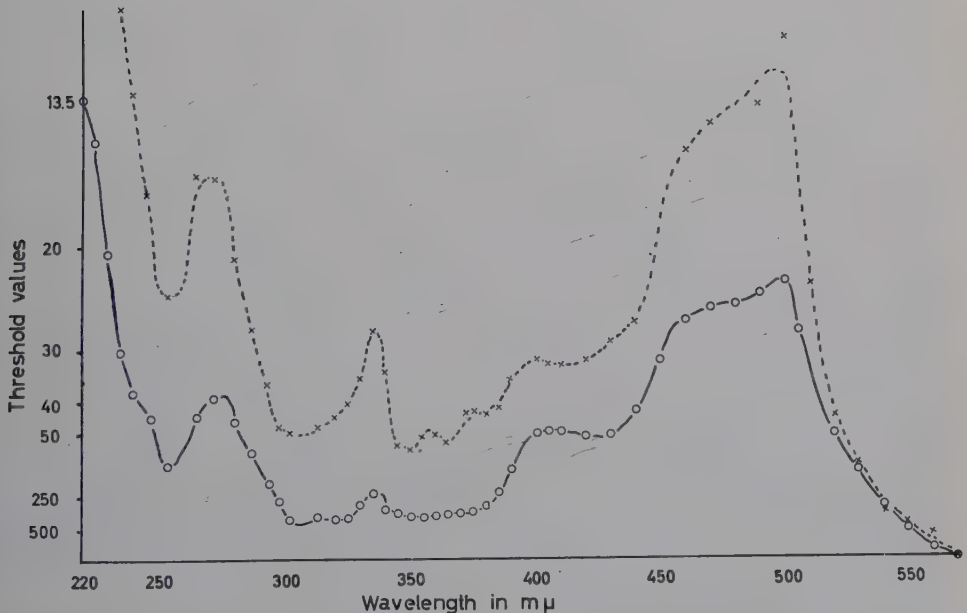


Figure 1. Action spectra of positive ----- and negative ——— phototaxis in *Platymonas subcordiformis* based upon threshold value determinations. Ordinate: lower limit of response in quanta/cm² sec. $\times 10^8$.

taxis when a reference light is applied difficult. The detail at 405 m μ show similarity to the maximum at 420 m μ in the action spectrum of *Euglena gracilis* (Bünning and Schneiderhöhn 1956). The shoulder at 450 was earlier reported to occur at 435 m μ .

In the ultraviolet a small maximum occurred at 335 m μ , another more prominent one at 275 m μ , and a minimum occurred at 255. From the last mentioned wavelength the action spectrum curve increased rapidly towards shorter wavelengths to 220 m μ . Below this wavelength no readings were taken.

Discussion

The action spectra of positive and negative phototaxis give the same peaks and valleys with about the same relative heights. This is the proof that the same photoreceptive pigment is involved in positive and negative topo-phototactic response in *Platymonas*. A distinct difference is however observed in the relative sensitivity in that the positively reacting cells responded to much lower radiation. This difference is not believed to be caused by internal screening. A discussion about this matter is given in an earlier publication (Halldal 1958). The difference occurred consistently during the whole experimental period and was observed within the same population when the response changed from negative to positive. It has not been possible to give an explanation of this.

These action spectra of positive and negative phototaxis in *Platymonas* strongly indicate that the photoreceptive pigment is a carotenoid combined with a protein which contains aromatic amino acids (For references about protein absorption see Takashima 1960). Due to the long conjugated double-bond chain of the carotenoid compounds one to three absorption bands are expected in the ultraviolet (Karrer and Jucker 1948). The ultraviolet action spectra indicate that the photoreceptive carotenoid has an *in vivo* absorption maximum at 335 m μ . The peak in the action spectrum at 275 m μ is not believed to correspond to absorption from a carotenoid but to the aromatic amino acids contained in the protein, though a carotenoid absorption band in this region may contribute somewhat to the action spectrum peak. In the visible region three absorption bands which are stronger than those in the ultraviolet are commonly found in the carotenoids. The action spectra of phototaxis reflect three such bands. One at 405 m μ , another around 450 m μ (shoulder), and a third at 495 m μ .

Riboflavin must be excluded as the photoreceptive pigment in phototaxis mainly because of its absorption characteristics in the ultraviolet. Riboflavin has a maximum around 370 to 380 m μ . No peak is observed in the action

spectrum at these wavelengths. The chief argument, however, is the extremely prominent absorption around 270 m μ , which is three times as high as the maximum absorption in the visible region.

The pterins generally contain a strong absorption band around 240 to 280 m μ and a weaker band at 340 to 380 (Sinsheimer 1955), and usually no detailed absorption band in the visible. These absorption characteristics have no resemblance to the action spectra of phototaxis. A carotenoprotein seems therefore to be the only possibility left.

The action spectra analyses that are performed have given a great many different action spectra, which in some cases differ considerably from each other. Generally the peak is observed around 475 and 495 m μ . The greatest deviation from this have been reported for negatively reacting *Euglena viridis* and in the chlorophyll-free and colourless form of this species which had action spectrum maximum at 410 m μ . (Bünning and Schneiderhöhn 1956, Gössel 1957). The experimental procedure applied in these experiments has been questioned by Halldal (1958, 1961) who concluded that: (1) experiments must be conducted in such a way that induced phototactic response changes are eliminated, as action spectra of induced phototactic response changes may differ considerably from action spectra of topo-phototaxis (Halldal 1960), (2) phobo-phototactic response must not be mixed with topo-phototaxis. A minimum requirement for this is that the cells have unrestricted opportunity to move in the radiation path, (3) colourless and bleached forms must be avoided as action spectra analyses with these give more information about absorption characteristics of the cells than pigments involved in phototaxis (see Halldal 1961). It is generally concluded that only the positively reacting chlorophyll-containing *Euglena gracilis* in the experiments of Bünning and Schneiderhöhn, give significant information about the pigment involved in photic orientation of this species.

A great deviation was also observed in the spectral sensitivity of *Prorocentrum micans* (Halldal 1958) which showed a maximum at 570 m μ and no further detailed characteristics. In these analyses the method with the reference beam was applied and the base of the curve was consequently eliminated. Due to their sluggish motion the dinoflagellates are not too well suited for the extremely precise responses that are needed to measure an action spectrum of phototaxis accurately. Some details might have been overlooked in the action spectrum of *Prorocentrum*, the peak position is however assumed to be correct.

Krinski and Goldsmith (1960) reported that the three keto-carotenoids echinenone, euglenanone, and hydroxy-echinenone were present in trace amounts in *Euglena gracilis*. One of these, hydroxy-echinenone, should be capable of binding to protein, with accompanying shifts of absorption spec-

trum to longer wavelengths. It is therefore theoretically possible that the photoreceptive pigment of phototaxis in *Euglena*, *Volvocales*, *Ulva*-gametes, and the dinoflagellates could be the same carotenoid and possibly hydroxyechinenone. Goodwin (1959) also assumed that the difference observed in the dinoflagellates *Goniaulax* and *Prorocentrum* is not significant as the pigments *in vivo* could exist as different carotenoproteins.

In the present discussion the assumption was made that the photoreceptor is a carotenoid combined with a protein. Another interpretation of the action spectrum is that the pigment and the protein are separated and that radiant energy absorbed by the protein is transferred to the pigment. In analogy with what is known from vision where a pigment of the same nature as a carotenoid (retinene) can exist in close association with protein molecule (opsin) the first possibility seems to be more probable.

The action spectra analyses performed on phototropism on *Phycomyces* sporangiospores in the ultraviolet show many features common to the action spectra of phototaxis presented above (Delbrück and Shropshire 1960 and Curry and Gruen 1959). In phototropism of *Phycomyces* action spectra peaks are recorded by Curry and Gruen at 370, 445 and a shoulder at 470 m μ . In the far ultraviolet for the negative response at 280, a minimum at 255 and from this wavelength increased activity toward shorter wavelengths. Delbrück and Shropshire recorded peaks at 280, 385, 455, and 485 m μ . The 280 m μ peak in Delbrück and Shropshire's experiments was 6.5 times as large as the corresponding peak in the growth spectrum. Otherwise the three spectra dealt with above show great similarities to one another. One may therefore assume that the same carotenoid, or related carotenoids, is involved in phototropism and growth in *phycomyces* and in phototaxis in flagellates. The minor changes that exist in peak positions and general shape may be due partly to attachments to different proteins and partly to internal screening.

Summary

The action spectra of positive and negative phototaxis in *Platymonas* show that the same photoreceptive pigment is involved in these two reaction types. In the far ultraviolet the algae are extremely sensitive to 220 m μ radiation. A minimum is observed at 255 m μ and a maximum at 275 m μ . This part of the action spectrum curve corresponds to the absorption spectrum of a protein which contains aromatic amino acids. In the near ultraviolet maximum occurred at 335 m μ , and in the visible region a small maximum at 405, a shoulder at 450, and a more prominent maximum at 495 m μ . The action spectrum curves in the near ultraviolet and the visible region cor-

respond closely to the absorption characteristics of certain carotenoids. It is concluded that a carotenoprotein is the photoreceptive pigment complex in photic orientation.

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Toxicity of Trichloracetates to *Chlorella vulgaris*

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Sodium and ammonium trichloracetates are useful herbicides for the control of certain grasses, but little is known about their mode of action. Trichloroacetic acid is frequently employed as a protein precipitant, but there is no evidence that the sodium salt acts similarly. Switzer (1957) observed that trichloroacetic acid, at concentrations of 0.02—0.05 M, inhibits oxygen uptake by mitochondrial preparations in the presence of succinate or pyruvate. Sen and Woodford (1953) demonstrated that both low and high concentrations of trichloroacetic acid depressed the elongation of pea root segments, but that intermediate concentrations accelerated growth. Mayer (1957) could find little evidence for a specific interaction between trichloroacetates and sulphydryl groups.

In view of this scanty evidence it was felt that the problem should be approached in a different way, using *Chlorella vulgaris* as test organism. A partial separation of effects on different physiological systems is possible, while analytical procedures are simpler than for higher plants.

Experimental Methods

1. Cultural techniques

Chlorella vulgaris was cultured on standard agar slopes in daylight at room temperature. The medium used for growth and uptake tests was a modified form of that used by Pearsall and Loose (1937), with a final pH of 6.1—6.3. Where applicable, glucose solution and varying amounts of the trichloroacetate were added.

Flasks to be illuminated were placed around a tungsten lamp to give a light intensity of 470 ft.-candles, at a temperature of $30 \pm 2^\circ\text{C}$. A 5 % mixture of sterile carbon dioxide in air was bubbled in when required. Tests on growth in the dark and all experiments on the uptake of glucose were carried out in a shaker at $30^\circ\text{C} \pm 0.5^\circ\text{C}$. The inoculum for tests on growth consisted of cells washed off agar slopes, and varied from 100 to 200 cells per mm^3 in different tests, counts being made with a Fuchs-Rosenthal haemocytometer.

In tests on sugar uptake, the cell concentration was about 35,000 per mm^3 , the cells being previously grown in glucose solution in the dark.

For respiratory studies, a conventional Warburg respirometer was employed in a darkened room at 30°C . Each flask contained approximately 3×10^8 cells, previously grown in darkness, and harvested after the end of the logarithmic growth phase. According to the type of investigation, the culture solution was the full growth medium, a phosphate buffer, or unbuffered saline containing 8.0 g sodium chloride and 0.4 g potassium chloride per litre of solution.

2. Chemical methods of analysis

Phosphorus compounds were separated into those which were acid-soluble (P_s) and acid-insoluble (P_n). The latter fraction consists mainly of nucleic acids, but also includes phosphoproteins. Preliminary tests showed that most of the P_s was extracted from *C. vulgaris* by the action of 10 % trichloroacetic acid for 1 hr. under ice-cold conditions, much smaller amounts being removed in two subsequent washings of 20 min. each at room temperature.

Determination of phosphate followed the method of Allen (1940). Inorganic orthophosphate was determined using aliquots withdrawn from acid-soluble fractions prior to perchloric acid digestion.

Nitrogen was determined by the micro-Kjeldahl method after sulphuric acid digestion, using the sample employed for dry weight determination, and carbohydrate was determined by the Hanes (1929) modification of the method of Hagedorn and Jensen.

Experimental Results

1. Toxicity tests

C. vulgaris was grown under three sets of conditions, namely, in glucose in darkness, and in the presence and absence of glucose in the light. Results typical of five similar experiments are shown in Figure 1. The dose I_{50} , of trichloroacetate needed to reduce the number of cells at any time to half that of the untreated flasks clearly varies with time. The problem of determining what are equi-effective concentrations when the response is curvilinear with time has confronted other workers (*e.g.* Blackman, 1952) but in the present study the best time for comparison lies between 3 and 5 days. A delayed response even to high concentrations of trichloroacetate when sugar is present limits the usefulness of short times of comparison, while after 4 or 5 days, shortage of sugar in the culture medium of cells grown in

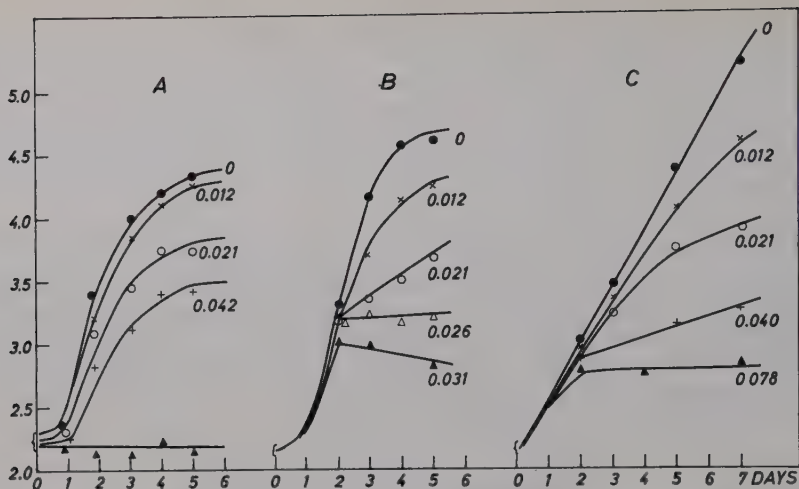


Figure 1. Effect of sodium trichloroacetate on the growth of *Chlorella vulgaris*. A. Photosynthetic conditions. B. Growth in glucose, illuminated. C. Growth in glucose in darkness. On the abscissa days after inoculation, on the ordinate log. number of cells per mm³. Sodium trichloroacetate concentrations in mol.

glucose in light becomes an additional factor limiting growth. Accepting five days as the standard time for comparison, I_{50} values, estimated from probit lines fitted by eye, were consistently 0.011 to 0.013 *M* trichloroacetate when glucose was present, and 0.018 *M* or more under photosynthetic conditions. Thus cultural conditions do not greatly affect the toxicity of trichloroacetates to cell multiplication.

An unexpected feature of growth in trichloroacetate solutions in the presence of glucose is apparent from Figure 1. Over a wide concentration range, cell division occurred at almost the normal rate for about 2 days, even at concentrations which by 5 days were highly inhibitory. The number of cells increased during this period from 200 per mm³ up to about 2,000 per mm³, corresponding to three or four divisions for each cell originally present. In experiments on the uptake of glucose reported in the next section, it was found that very much higher concentrations of trichloroacetate still allow some cell division for a limited period of time. Initial starvation of the cells by keeping them in the dark for 5 days in distilled water did not diminish this tendency to divide.

2. Chemical analyses on cells grown in trichloroacetate solution

Cells grown in the presence of varying concentrations of sodium or potassium trichloroacetate were separated, washed, and divided into samples of

Table 1. *Effect of sodium trichloracetate on the percentage composition on a dry weight basis of C. vulgaris growing under photosynthetic conditions.*

Composition of inoculum		% Phosphorus			P_s/P_n	% Nitrogen Total	% Carbo- hydrate, hydrolysable
		acid-insoluble (P_n)	acid-soluble (P_s)	Inorganic ortho-phosphate			
Composition of inoculum		0.45	0.18	—		3.8	21
Sodium trichloracetate M	Relative change in cell number, control=100 at 5 days						
0	0	2.2	0.36	0.24	0.16	8.3	22
0.0043	+ 9	2.2	0.40	0.28	0.18	8.4	21
0.0086	— 69	1.6	0.60	0.32	0.38	9.0	22
0.032	— 87	1.2	0.83	0.28	0.69	9.5	22
0.072	— 95	0.8	0.98	—	1.22	9.9	24
0	0	2.6	0.54	—	0.21	8.4	—
0.0068	— 57	2.5	0.88	—	0.35	8.6	—
0.017	— 73	1.8	0.60	—	0.33	8.5	—
0.021	— 74	1.3	1.10	—	0.85	8.2	—
0.032	— 96	1.0	1.50	—	1.50	8.4	—

Table 2. *Effect of sodium and potassium trichloracetate on the percentage composition on a dry weight basis of C. vulgaris growing in light or darkness in glucose.*

Conditions	Trichlor-acetate M	Relative change in cell number, control=100 at 5 days	% Phosphorus		P_s/P_n	% Nitrogen Total	% Carbo- hydrate hydrolysable
			acid-insoluble (P_n)	acid-soluble (P_s)			
Light, potas-sium salt	0	0	1.41	0.44	0.31	3.40	56
	0.013	— 56	1.25	0.56	0.45	4.51	56
	0.017	— 90	0.70	0.80	1.14	5.95	48
Light, sodium salt	0	0	1.90	0.20	0.11	3.42	—
	0.0084	— 8	1.68	0.24	0.14	3.50	—
	0.012	— 56	1.38	0.41	0.30	—	—
	0.017	— 80	1.36	0.50	0.37	6.78	—
	0.021	— 94	1.32	0.90	0.68	—	—
Darkness, sodium salt	0	0	0.60	0.095	0.16	3.20	—
	0.0063	— 52	0.57	0.124	0.22	3.19	—
	0.0084	— 62	0.50	0.118	0.24	3.09	—
	0.0125	— 80	0.55	0.155	0.28	3.56	—

suitable size. The percentage contents on a dry weight basis of acid-soluble (P_s) and acid-insoluble (P_n) phosphorus, nitrogen and hydrolysable carbohydrate are given in Tables 1 and 2. In three other experiments carried out on cells grown under photosynthetic conditions, the ratio P_s/P_n increased with trichloroacetate concentration in a similar way to those recorded in Table 1. Owing to the fact that cells from replicated flasks had to be bulked prior to analysis to provide sufficient experimental material, a full statistical check on these analytical figures was not practicable. Duplicates were sometimes possible for the controls and the lower trichloroacetate concentrations, and phosphate determinations on them seldom differed by more than 5 %. The consistent trend in P_s/P_n noted in Tables 1 and 2 is therefore important.

3. Effect of trichloroacetate on the uptake and utilization of glucose

In four experiments, cell populations of 35,000—45,000 cells per mm^3 were employed. The concentration of glucose varied in different tests from 0.1 to 0.5 %, and the duration of the tests from 5 to 10 hours. Concentrations up to twice the I_{50} for cell division had no effect on the rate of uptake of external glucose. Uptake was depressed at concentrations 6—20 times the I_{50} , but it still proceeded almost linearly with time until all the glucose in the external solutions had been absorbed.

Analyses of the cells before and after these tests were based primarily on a constant volume of the cell suspension. In this way any gain of cell substance during the course of the experiment, whether by cell division or cell enlargement, is directly apparent. Even at high concentrations of trichloroacetate, very considerable synthesis of insoluble carbohydrate accompanied glucose utilization. Thus, before shaking for 10 hours with glucose solution, the carbohydrate found within the cells was 6.2 mg. per 10 ml. of cell suspension. Afterwards, the corresponding figures were 19.2 mg. for the untreated cells, and 14.2 mg. for cells shaken in the presence of 0.215 *M* sodium trichloroacetate. During the 10 hours of shaking, the number of cells per mm^3 increased by 77% in the controls, and by 42 % in the presence of 0.215 *M* sodium trichloroacetate.

In two experiments the glucose taken up was compared with the quantity of insoluble carbohydrate formed in a standard volume of cell suspension during the same period. This enabled an estimate to be made of the effect of trichloroacetate on the net synthesis from glucose of hydrolysable polysaccharide. In both series, approximately 0.1 *M* trichloroacetate increased slightly the percentage of glucose respired or converted to non-carbohydrate material — from 53 to 59 % in the first, and from 48 to 52 % in the second test.

Table 3. *Dry weight of algal cells, and their nitrogen and phosphorus contents, in 10 ml. algal suspension before and after shaking with glucose.*

Conditions	Sodium trichloroacetate <i>M</i>	Dry weight mg.	Total N mg.	P _n mg.	P _s (total) mg.	P _n /N
A. Unstarved cells						
Before shaking	—	23.4	0.74	0.181	0.021	0.25
After shaking	0	29.9	1.06	0.224	0.021	0.21
for 6 hrs.	0.0086	31.0	1.17	0.230	0.023	0.21
	0.021	—	—	0.221	0.023	—
	0.065	29.4	0.95	0.201	0.023	0.21
	0.215	29.6	0.89	0.189	0.028	0.21
B. Glucose-starved cells, kept in dark						
Before shaking	—	14.4	0.74	0.192	0.023	0.26
After shaking	0	32.6	1.32	0.252	0.038	0.19
for 10 hrs.	0.215	27.9	1.17	0.233	0.035	0.20

The effect of trichloroacetate on the assimilation of phosphorus and nitrogen during the course of glucose uptake is shown in Table 3 for two experiments. In the controls of both starved and normal cells, the ratio P_n/N falls during uptake. This fall presumably reflects a shortage of nitrogen in the growth medium at the time the cells were harvested. The change in this ratio during the experiment is not altered by the presence of even large doses of trichloroacetate, despite the very different amounts of phosphorus assimilated at different concentrations.

4. Respiratory studies

Sodium trichloroacetate, prepared from the purest grades of trichloroacetic acid commercially available, appeared to stimulate the endogenous respiration of *C. vulgaris*. However, if the trichloroacetate was destroyed by prolonged boiling in alkaline solution, the neutralized solution still stimulated respiration, perhaps because of the presence of a trace of acetate or other metabolite. After 20 hr., the apparent stimulation had disappeared, but even after 48 hr., respiration in 0.08 *M* trichloroacetate had not fallen below that in the untreated flasks. No damage to the respiratory mechanism of the algal cells could therefore be detected after the cells had been in prolonged contact with trichloroacetate concentrations seven or more times the I_{50} for cells growing in glucose in darkness.

Small quantities of authentic sodium acetate were added to flasks containing varying amounts of trichloroacetate. Respiration did not decrease until the trichloroacetate concentration approached 0.3 *M*, and even at 0.5 *M* it was

Table 4. *Effect of trichloracetate on respiration associated with glucose and ammonia uptake.*

Addition to algal cells in phosphate buffer in flasks	Contents of side arm	Initial respiration $\mu\text{l/hr.}$
Nil	Nil	47
0.2 M sodium trichloracetate	Nil	65
Nil	15 mg. glucose	253
0.2 M sodium trichloracetate	15 mg. glucose	283
Nil	Water	21
Nil	1.5 mg. NH_4NO_3	128
0.08 M sodium trichloracetate	Water	33
0.08 M sodium trichloracetate	1.5 mg. NH_4NO_3	140

still above the endogenous level. Moreover, the eventual decrease was probably a non-specific effect related to the high osmotic concentration, for the addition of 0.38 M sodium chloride to 0.12 M trichloracetate reduced the respiration a little more than the addition of 0.38 M trichloracetate. Similarly, (Table 4), the respiration associated with the uptake of glucose was unaffected by massive doses of trichloracetate. A third metabolic activity shown by *Chlorella* cells starved of nitrogen is the increased oxygen uptake which occurs when the cells are provided with ammonium compounds in the external medium (Fogg 1953). Oxygen uptake in the presence of ammonium nitrate was not reduced by 0.08 M trichloracetate, the highest concentration tested.

Cells which had been suspended in phosphate buffer containing trichloracetate were analysed for alcohol-soluble and insoluble carbohydrate. At high levels of trichloracetate, a sharp rise in the soluble carbohydrate took place, accompanied by a corresponding fall in polysaccharide content. The ratio of soluble to insoluble carbohydrate rose to almost twice the value of the corresponding quantity for untreated cells as the trichloracetate concentration increased to near the osmotic limit of 0.3 M. No rise was, however, observable at concentrations below about 0.05 M.

In contrast to the respiratory stimulation which occurs when commercial trichloracetate in concentrations up to about 0.3 M was added to cells in the unbuffered saline, free trichloracetic acid above 0.01 M stopped respiration completely. The respiration of 6-day old cells was reduced to about one-half the control value within a few minutes by 0.006 M trichloracetic acid (saline suspension medium, pH 2.7), older cells being affected by concentrations as low as 0.001 M. Respiration is inhibited by trichloracetic acid more rapidly than it is by mineral acids at comparable pH values, and therefore the toxicity of free trichloracetic acid cannot be entirely attributed to the direct effect of hydrogen ions.

Discussion

Trichloroacetic acid has a pK value of 0.4—0.7. Thus only 0.2—0.5 % exists in the undissociated form at pH 3, and less than 0.01 % at pH 6. The somewhat low toxicity of trichloroacetate to metabolic activities within the cell may be due to poor penetration, if trichloroacetate ions enter less readily than undissociated molecules. Nevertheless, Switzer (1957) found that 0.02 *M* trichloroacetic acid (at unstated pH) was required to reduce the respiration of isolated soya-bean mitochondria, indicating either that the inherent toxicity of trichloroacetate to respiration is low, or that permeability barriers other than those at the cell wall-cytoplasm interface may be of importance. Whatever the concentration gradient across the cell, some metabolic factors proved in the present study to be far more susceptible to the internal trichloroacetate than others.

The I_{50} value at 5 days, namely, 0.01—0.02 *M* trichloroacetate, is a measure of the threshold where toxic effects appear, the average number of divisions being reduced by approximately one-eighth at this concentration. The most striking chemical change observed at and above this concentration was an alteration of the balance of acid-soluble to acid-insoluble phosphorus (P_s and P_n) in favour of the soluble phosphate (Tables 1 and 2). This shift does not necessarily take place when the growth of cells is retarded by a poison. In media containing fluoride, for example, unpublished results show that P_s and P_n fall almost proportionately as the concentration increases from zero to 0.02 *M*. It is therefore possible that the primary effect of trichloroacetate may be upon phosphorus metabolism, and Table 3 demonstrates that the amount of phosphorus entering the P_n fraction of growing cells in unit time is much reduced in the presence of trichloroacetate. The rise in P_s may be due to an increase in the 'organic' rather than inorganic phosphate (Table 1) which, in conjunction with the fall in P_n , suggests that trichloroacetates may block the synthesis of nucleoproteins.

The total nitrogen content of such cells as are able to grow in trichloroacetate is little affected, except perhaps, in glucose in the presence of light, at concentrations highly inhibitory to growth (Tables 1 and 2), but the rate of uptake of nitrogen (N) by a *Chlorella* suspension decreases with increasing concentration (Table 3). However, the rate of formation of P_n similarly decreases, the ratio P_n/N being independent of trichloroacetate concentration. The explanation may be that the effect on nitrogen metabolism, and hence on the number of cells formed in a given time, is due indirectly to limited synthesis of ribonucleic acid.

In contrast to these effects at external concentrations as low as 0.01 *M*, sodium trichloroacetate is no more toxic to respiration than is sodium chlo-

ride. For both salts, 0.3—0.5 *M* solutions are required to inhibit respiration. While lack of penetration may help to explain this tolerance, much lower external trichloracetate concentrations cause changes in other internal systems. Enzymes associated with carbohydrate metabolism are therefore relatively insensitive to such trichloracetate as enters the cell. There is evidence, reviewed by Taylor (1957), that phosphorylative enzymes concerned with the uptake of sugar are located on the outer surface of certain cell membranes, including that of *Scenedesmus*. These enzymes, if operative in *Chlorella*, are apparently not affected by high external concentrations of trichloracetate (Table 4). Similarly, it is apparent from respiratory studies that alkali metal salts of trichloroacetic acid, unlike the acid itself, do not rapidly destroy the membrane boundary of the cell.

While caution is needed in attempting to relate observations on *Chlorella* to the herbicidal effects of trichloroacetates in the field, there is little need to emphasize that photosynthetic and phosphorylative systems are broadly similar, and nucleic acids probably have similar functions, in *Chlorella* and in higher plants. A study of the effect of trichloroacetate on phosphorus metabolism in higher plants might, therefore, be of interest.

Summary

Some toxic effects of trichloroacetates have been investigated using a strain of *Chlorella vulgaris* which grows readily in the dark with glucose as the source of carbon. Cell division was retarded to the same extent by similar concentrations of toxicant under photosynthetic and non-photosynthetic culture conditions. At concentrations exceeding approximately 0.01 *M*, cell division was retarded, and the proportion of phosphorus compounds which were 'acid-soluble' increased in the growing cells.

The uptakes of external glucose, phosphorus and nitrogen by a concentrated suspension of *Chlorella* were all depressed by concentrations above 0.06 *M*, such that the final ratio of cell phosphorus to nitrogen was independent of the trichloroacetate concentration. Respiration, as measured by uptake of oxygen, was not reduced by concentrations of trichloroacetate below 0.4 *M*, above which non-specific osmotic effects were probably the cause of respiratory failure. In contrast, 0.001 *M* trichloroacetic acid decreased respiration of old cells appreciably in an unbuffered medium.

It is a pleasure to record my thanks to Professor G. E. Blackman and Dr. R. T. Wedding for advice and criticism, and to Miss J. Carrinci for valuable technical assistance.

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The Influence of Growth Substances on the Formation of Shoots from Aspen Roots

By

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Aspen (*Populus tremula*) is known to propagate abundantly by shoots emerging from the roots (root suckers). Due to the wide distribution of this species, its suckering power creates difficult problems in the silviculture. In recent years various phytocides, primarily 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), have been used to eradicate aspen. The effectiveness of these chemicals in preventing aspen regrowth is, however, varying. More knowledge of the responses of aspen to growth-regulating phytocides is needed in order to secure a more rational use of these means.

The root system of aspen is characterized by long, rather slender, horizontal roots a few centimeters below the soil surface. Day (1944) investigated the root system of the closely related quaking aspen (*Populus tremuloides*) and Joachim (1953) made extensive root investigations on several *Populus* species. Buell and Buell (1959) also relate some interesting observations regarding the extension of aspen roots. From isolated segments of aspen roots, kept under appropriate temperature and moisture conditions, suckers rapidly develop. This phenomenon has been exploited in the various methods of vegetative propagation of aspen (Wettstein 1940, Kuchlenz 1958, for further references see Børset and Haugberg 1960). The investigation reported in this paper is concerned with the effects of growth substances, in the first place phenoxy compounds, on isolated segments of aspen roots.

Methods and Results

The root material used in the present investigation was obtained from forest stands of aspen, mainly at Bogesund near Stockholm. The selected root material was protected against dry air and used in the experiments within a day or two. Only roots with a diameter between 6 and 15 mm. were used. The roots were cut into 15 cm. long segments with a pair of cable pincers, rinsed with tap water and randomly distributed between the various treatments. The number of segments used in each treatment varied between 5 and 10 in different experiments.

The substances used in the experiments were dissolved in phosphate buffer solution (1.5 mM KH_2PO_4 , 0.5 mM Na_2HPO_4 , pH 6.5). The root segments in each sample were placed in a Petri dish with 250 ml. of the test solution for 24 hours. After the treatment the roots were rinsed with water, dipped in Bordeaux solution (2 gm. $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ and 2 gm. CaO per l.) and placed vertically in plastic pots (height 13 cm., diameter 10 cm.) with the lower end (2 to 4 cm.) dipping into water. In order to avoid too rapid evaporation polyethylene sacks with perforations for aeration were placed over the pots. The material was stored for about four weeks in light from fluorescent tubes at $25 \pm 2^\circ\text{C}$. During this period observations were made at least once a week.

In the control (treated with buffer solution) shoots began to emerge after about one week. After four weeks several shoots had appeared on most of the control root segments, sometimes ten to twenty on each. Occasionally there were visible buds or other protuberances on the roots, from which shoots emerged. Most of the shoots, however, grew out from parts of the root surface with no observable differences from other parts of the root. Often several shoots emerged close together, presumably from some sort of callus tissue. Also from wound callus growing at the cut ends of the segments shoots may occasionally develop, but this type of shoot formation is of secondary importance. The season has a strong influence on the capacity for shoot formation. Thus there was a much more abundant shoot formation on roots excavated from October to May than during the summer. This appears to be due to the decrease in carbohydrate reserves of the roots connected with the leafing and growth of new shoots (cf. Woods 1955).

Table 1 shows the effect of treatment with some growth substances on shoot formation. As a comparison also results obtained with the phytotoxic substance aminotriazole have been recorded. Table 1 is based on five independent experiments with 2,4-D and 2,4,5-T and on at least two with the other substances. These experiments were carried out from October 1959 to May 1960. Some tests with 2,4-D and 2,4,5-T were also made during the summers of 1959 and 1960. At these times the root segments showed somewhat greater sensitivity to the inhibitory effect of the substances, $10^{-5} M$ 2,4,5-T giving complete and $10^{-5} M$ 2,4-D strong inhibition of shoot formation. The signs of Table 1 refer to the number of shoots developed, and no consideration is paid to the influence on growth of the shoots after emergence. Such an influence was found only after treatment with $10^{-3} M$ TBA. This substance inhibited the growth of the developed shoots besides causing malformations of the leaves. In addition to the substances listed in Table 1

Table 1. *The effect of growth substances and aminotriazole on the shoot formation from aspen roots.* Root segments were treated with buffered solutions of the substances for 24 hours and the subsequent shoot growth followed for four weeks. Symbols: +++ no inhibition; ++ slight inhibition; + strong inhibition; — complete inhibition.

Substances	Concentrations <i>M</i>				
	10^{-5}	3×10^{-5}	10^{-4}	10^{-3}	10^{-2}
2,4,5-trichlorophenoxyacetic acid (2,4,5-T)	+	—	—	—	—
2,4-dichlorophenoxyacetic acid (2,4-D)	++	+	—	—	—
2-methyl-4-chlorophenoxyacetic acid (MCPA)	++	+	—	—	—
4-chlorophenoxyacetic acid (4-ClPOA)	+++	++	+	—	—
1-naphthylacetic acid (NAA)	+++	+++	++	—	—
3-indolylacetic acid (IAA)	+++	+++	++	+	—
2,3,6-trichlorobenzoic acid (TBA)			+++	+	
3-amino-1,2,4-triazole			+++	+	—

a number of other compounds were tested in preliminary experiments. From these tests it may be mentioned that the racemic forms of α -propionic acids corresponding to 2,4-D, 2,4,5-T, and MCPA all had weaker activity than the acetic acids. On the other hand, γ -(2-methyl-4-chlorophenoxy)-butyric acid had about the same effect as MCPA.

Besides the influence on shoot formation the substances had several other effects. One of these was some sort of general toxicity shown by the phenoxy substances at higher concentrations. The symptom of this toxicity most easily observed was that the living tissue in the bark turned brown after one or two weeks. In this connexion the segments were heavily attacked by microorganisms. Also in causing this effect 2,4,5-T showed a markedly stronger activity than the other phenoxy substances investigated. Already in the treatments with 10^{-4} *M* 2,4,5-T the necrotic symptoms gradually developed, while 2,4-D and MCPA must be applied at least in 10^{-3} *M* to cause the same response. Still weaker activity was shown by 4-ClPOA, and the other substances of Table 1 exhibited very little tendency of causing necrosis and stimulating microbial decomposition of the tissue in the concentrations tested.

Another response obtained after treatment with phenoxy substances was swelling or proliferation of the living parenchyma in the bark of the root segments. The swellings were developed during the first 10 to 15 days after treatment. The periderm was often ruptured and the proliferated tissue soon began to decompose. No swellings were obtained in summer, a fact that may be connected with the low carbohydrate content of the roots during this season. In inducing proliferation the most active substances were 2,4-D, MCPA, and 4-ClPOA. By treatment of the segments with these substances for 24 hours somewhat higher concentrations were required to induce this

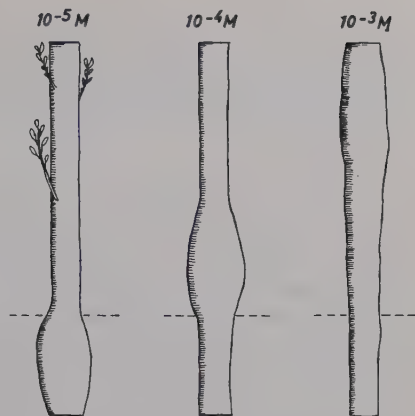


Figure 1. Typical swellings on root segments of aspen which had been treated for one week with 2,4-D. Only the part of the segments below the line of short dashes was in contact with the solution. To eight segments 200 ml. solution was used.

response than to inhibit shoot formation, $10^{-3} M$ giving the most pronounced result. At higher concentration (10^{-2}) no swellings occurred. In contrast 2,4,5-T caused only slight swellings and in inducing this response only low concentrations ($3 \times 10^{-5} M$) were effective. The impression given by these results is that the toxicity of higher concentrations of the substance prevents proliferation and that the effective concentration range is especially narrow for 2,4,5-T. This dependence of the swelling on concentration is more clearly illustrated by one experiment in which the treatment was extended to one week and only the lower ends of the segments were brought into contact with the test solution. The appearance of the segments ten days after the beginning of the treatment with 2,4-D is shown in Figure 1. Evidently during these experimental conditions there was established a concentration gradient in the segments with the highest concentration of the growth regulator in the tissue in direct contact with the solution. The lower concentrations of 2,4-D used (10^{-5} and $3 \times 10^{-5} M$) caused swellings only in the root parts which had been in contact with the solution. Swellings were caused by $10^{-4} M$ in the part of the segment located immediately above the solution surface while the part beneath the surface was unaltered. With $10^{-3} M$ the most pronounced swellings were obtained in the upper parts of the segments. In this experiment MCPA gave essentially the same result as 2,4-D, while 2,4,5-T also in this case caused slight swellings only at the lower concentrations tested.

Neither IAA or NAA, after treatment with $10^{-2} M$ or lower concentrations, caused proliferation or necrotic symptoms of the kind described above. These substances, however, stimulated formation of wound callus at the cut surfaces of the segments and especially NAA was active in inducing growth of lateral roots. To some degree also 2,4-D and MCPA showed the same

effects when used in low concentrations. Thus root growth was often observed after treatment with 10^{-4} M 2,4-D. The substance most active in stimulating callus growth was TBA. Especially after treatment with 10^{-3} M of this substance there was vigorous callus formation.

Discussion

The knowledge of the physiological processes controlling the development of shoots from roots of suckering species is scanty. Emery (1955) found that IAA treatment inhibited bud formation in root cuttings of *Chamaenerion angustifolium*, while callus growth and root formation were stimulated. Several other investigators, cited by Emery, have suggested that formation of buds on roots is controlled by local auxin concentrations in the tissue. As pointed out by Torrey (1958), it is probable that the bud initiation is dependent upon a number of specific chemical factors. One of these appears to be a kinetin-like factor, since Danckwardt-Lillieström (1957) found that kinetin induced shoot formation from isolated roots of *Isatis tinctoria*. The results obtained by Skoog and Miller (1957) with isolated tobacco stem tissues indicate that bud formation is regulated by the balance between IAA and other factors such as adenine and kinetin.

The present results show that auxins inhibit shoot formation from aspen roots. The high activity of phenoxy substances may be due partly to a higher persistence of these substances than of IAA and NAA. Besides there is evidence that the toxicity of especially 2,4,5-T may interfere with the auxin activity of the substance. In auxin tests 2,4,5-T shows usually weaker activity than 2,4-D and MCPA, and its high activity in the inhibition of shoot formation would be unexpected if it were only a manifestation of the auxin properties of the substance. A comparison of the responses induced by the relatively non-toxic auxins IAA and NAA with those induced by the phenoxy substances indicates that the true auxin activity of the latter is revealed only at the lower concentrations used. While IAA and NAA stimulated wound callus growth and induced root formation, 2,4,5-T gave none of these responses. MCPA and 2,4-D were intermediate between the former auxins and 2,4,5-T in this respect. It may, however, be noted that there occasionally occurred lateral root formation after treatment with 10^{-4} M 2,4-D, a concentration which completely inhibited shoot formation. This is consistent with the relationship between shoot and root formation after auxin treatment found by Emery (1955), and it indicates that the inhibition of shoot formation caused by 2,4-D was due to the auxinic properties of the substance. For 2,4,5-T, on the other hand, it is evident that the toxic properties

are of greater importance. The proliferation abundantly obtained after treatment with 10^{-4} and 10^{-3} M 2,4-D, MCPA and 4-ClPOA was rather weak in 2,4,5-T-treated roots and occurred only at low concentrations. This indicates that the toxicity of 2,4,5-T at higher concentrations also limited the proliferation.

The results suggest that there is no correlation between auxin activity and phytotoxic properties of the phenoxy compounds. The nature of the toxicity shown especially by 2,4,5-T but also by other phenoxy substances in higher concentrations needs further investigation. Probably this toxicity is of decisive importance in the use of phenoxy substance for the eradication of woody plants as 2,4,5-T has been found for several species to be superior to 2,4-D and MCPA (Tam 1947, Peavy and Burns 1959).

One important result of the investigation is that it reveals the high sensitivity of aspen roots to phenoxy substances. After treatment of the leaves or stems of aspen with phenoxy compounds, translocation of only minute quantities of the substances into the roots should cause such damage to the roots that they lose their power to form shoots. Consequently, translocation of phenoxy substances into the roots could be followed by taking root segments from treated trees or bushes and storing these under conditions permitting shoot development. Work along this line is in progress.

Summary

Root segments of aspen were treated with growth substances and subsequently grown in light for four weeks under such conditions that root suckers were abundantly formed from the control segments. The following responses were obtained:

1. Inhibition of shoot formation. Phenoxy substances were more active than IAA, NAA and IBA in inducing this inhibition. Most effective was 2,4,5-T. The sensitivity to the phenoxy substances was greatest in the summer when the ability of the segments to form suckers was less than in other seasons.

2. Gradually appearing necrotic symptoms connected with rapid microbial decomposition induced by phenoxy substances in higher concentrations. This response was caused by 2,4,5-T in considerably lower concentrations than by the other substances.

3. Proliferation of tissues in the bark. The proliferation was induced only by a rather narrow concentration range of the phenoxy substances, 2,4-D, MCPA and 4-ClPOA being the most active.

4. Stimulated wound callus growth mainly obtained after treatment with TBA, NAA and IAA.

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Apparent Free Space and Surface Film Determined by a Centrifugation Method

By

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The term "apparent free space" (A.F.S.) was originally used by Hope and Stevens (1952) to describe the portion of the plant tissue into which substances in solution apparently move by free diffusion. Thus A.F.S. ought to be an expression for a certain volume of the tissue and not an amount of a substance or only an index. Since this first account and determination of A.F.S. many workers have described methods for its determination mainly in roots of higher plants. (Butler 1953, 1959, Epstein 1955 a, b, 1956, 1960, Kramer 1956, 1957, Briggs and Robertson 1957, Burström 1957, Kylin and Hylmö 1957, Robertson 1958, Brouwer 1959, Laties 1959, Bernstein and Nierman 1960, Pettersson 1960) but also in leaves (Kylin 1957, 1960) in storage tissue (Briggs, Hope, and Pitman 1958) in algae (Eppley and Blinks 1957, Bergquist 1958, Mac Robbie and Dainty 1958, Dainty and Hope 1959) and in bacteria (Cowie and Roberts 1955).

The A.F.S. values which have been given in the literature differ so strongly — from about 3 per cent up to 50 per cent or higher — that it is very probable that this variation is not always caused by differences in the plant material employed. More probably it is due to differences in the determination methods and in the ideas of the A.F.S. We think that it is essential to follow the original definition. Part of the plant tissue is postulated to be available for free diffusion of substances from the surrounding medium. The aim is then to determine the real magnitude of the free space and to establish what anatomical parts of the cell are included.

Levitt (1957) has critically reviewed the A.F.S. determination methods. He finds that the great variation of the A.F.S. values of plant roots may be caused by a surface film about $20\ \mu$ thick which has been included. Such a surface film should strongly raise the A.F.S. value of thin roots as compared with that of thicker roots. The correct A.F.S. value of all investigated roots is according to Levitt about 8 per cent of the root volume. This should suggest that those parts of the root which are available to free diffusion are represented by the cell walls.

The investigation presented here is an experimental attempt to determine the existence and magnitude of the surface film postulated by Levitt (1957) for theoretical reasons.

Materials and Methods

Wheat of Weibulls "Eroica" variety was used. The seeds were germinated by soaking in tap water overnight and then spread out on moist filter paper in Petri dishes. The dishes were placed in a dark room at 22°C . After two days the seedlings were mounted on perspex holders each holding 14–17 plants. Four such holders were cultivated in 1-litre glass beakers containing 800 ml. standard nutrient solution of the following composition: 1 mM $\text{Ca}(\text{NO}_3)_2$, 1 mM KH_2PO_4 , 0.5 mM Na_2HPO_4 , 1 mM KCl , 0.5 mM MgSO_4 , 0.01 mM Fe-citrate , 1 μM MnSO_4 , 1 μM H_3BO_3 , pH about 6.5.

Water losses were made up for every day and the nutrient solution was totally renewed every second day. Continuous aeration of the solution was arranged. The temperature was kept at $22 \pm 1^{\circ}\text{C}$. Light was supplied continuously by two 500 watt incandescent bulbs at an intensity of approximately 9000 lux.

The experiments were performed when the plants had grown for 5 days on nutrient solution. In some experiments (longitudinal centrifugation) where the roots for experimental reasons could not be longer than about 8 cm they were about 4 days old. Kylin and Hylmö (1957) have shown that A.F.S. of wheat roots is constant in this age interval.

The experiments with transverse centrifugation (exp. 1) were performed with bundles of excised roots in 250 ml glass beakers containing 100 ml standard nutrient solution labelled with radioactive sulphate (about 100 $\mu\text{C/litre}$). Each beaker contained two root bundles weighing about 2 grams together.

The roots from one perspex holder were cut off and collected into a bundle by means of a thin nylon string. Lying on the bottom of a brass net basket at 10 cm distance from the centrifuge axis the roots were run at 1000 revolutions per minute (corresponding to a centrifugal force of 110 g) for 30 seconds. They were immediately weighed to the nearest 0.01 gram and this weight was called *standard weight*. The roots were put down into the radioactive solution for a certain time. After the end of the experimental time the roots were freed from excess solution by a new centrifugation now at different R.P.M. in the range 1000–3500 always for 30 seconds. The root weight after this second centrifugation is named *final weight*.

The experiments with longitudinal centrifugation (exp. 2) were modified in the following way. The shoots were cut off while the roots were left on the holder.

The roots were gently blotted with filter paper and the holder with roots was put into radioactive solution. After the experimental period the roots were centrifuged still mounted on the holder. Then the roots were cut off and weighed (final weight). The standard weight was not determined for technical reasons.

In all centrifugations the mouth of the tube was covered by a rubber cap to prevent evaporation. The liquid that was thrown away was absorbed by cotton in the bottom of the tube.

For the A.F.S. determinations the experiments were performed as time series from which the analytic values were plotted in a diagram with the axes time and radioactivity/gram roots. The amount of sulphate taken up in A.F.S. (initial uptake) was obtained by extrapolating to zero time (Kylin and Hylmö 1957). A.F.S. is expressed in per cent of the root weight (=per cent of the root volume since the density of wheat roots is about 1) according to the following equation:

$$\frac{\mu \text{ mol SO}_4^{2-} \text{ initial uptake/g fresh weight}}{\mu \text{ mol SO}_4^{2-}/\text{ml ambient solution}} \cdot 100 = \text{A.F.S. (ml/100 g. fresh weight)}.$$

Three experimental periods were used at first; 30, 120 and 240 minutes. The second phase of the sulphate uptake proved to increase rectilinearly with time in this interval as found by Kylin and Hylmö (1957). In the later experiments only two periods were used; 30 and 150 minutes.

The experiments were performed in a refrigerated room at $4 \pm 1^\circ\text{C}$ in order to eliminate errors due to metabolic ion uptake as much as possible. Test solutions and plant material were placed in the refrigerated room about 4 hours before the experiments began. During this time as well as during the experiments air was bubbled through the solutions.

The sulphur analyses were made according to a method described by Kylin (1953) and Kylin and Hylmö (1957). They involve combustion by wet oxidation of the plant material, precipitation of the radioactive sulphur as BaSO_4 and GM-counting on samples of standard area and weight.

All experimental values used for the extrapolation of A.F.S. are means of two independent determinations on different samples.

Experiments

1. *Transverse centrifugation*

In four series (one was not complete) the roots were centrifuged lying on the bottom of a brass net basket at a distance of 10 cm from the centrifuge centre. The force was working transversely more or less at right angles to the length axis of the roots.

The results of these experiments where A.F.S. is calculated in per cent of the final root weight are presented in Figure 1 A. With increasing force A.F.S. asymptotically decreases from about 20 per cent at 700 R.P.M. (50 g) to about 11 per cent at 3500 R.P.M. (1400 g) the highest velocity that was

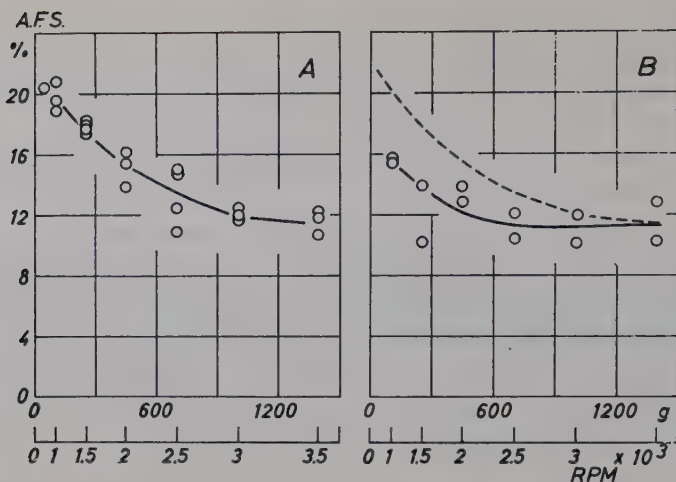


Figure 1 A. *A.F.S. of excised wheat roots after removing excess solution by transverse centrifugation at different velocities.* On the vertical axis A.F.S. in per cent of the final weight. The roots were centrifuged lying at the bottom of a basket 10 cm from the centre. On the horizontal axis the centrifugal force at this distance. The experimental velocities of the centrifuge (R.P.M.) are given on the scale below.

Figure 1 B. *A.F.S. of excised wheat roots after removing excess solution by longitudinal centrifugation at different velocities.* On the vertical axis A.F.S. in per cent of the final weight. The roots (about 8 cm long) were centrifuged mounted on a perspex holder. The distance from the centre to the holder was about 2 cm and to the root tips about 10 cm. On the horizontal axis the centrifugal force at a distance of 10 cm. Broken line is the curve from Figure 1 A.

used. This favours Levitt's assumption that many A.F.S. determinations include a surface film.

When filter paper was used to remove the excess solution A.F.S. was determined to about 18 per cent as has been reported by Kylin and Hylmö (1957) for excised wheat roots.

2. Longitudinal centrifugation

Two series were performed where the roots were centrifuged longitudinally still mounted on the perspex holders. The centrifugal force was working along the roots. The roots were about 8 cm long, and the distance from the root tips to the centrifuge centre was about 10 cm. The results are presented in Figure 1 B where the given centrifugal forces are calculated at a distance of 10 cm from the centre.

In these experiments A.F.S. also decreased with increasing centrifugal force. At velocities of 2500—3500 R.P.M. the A.F.S. was 11 per cent or the same as was found at the highest velocity 3500 R.P.M. in the transverse centrifugations. At lower velocities A.F.S. is throughout smaller after a longitudinal centrifugation than after a transverse. The surface film is thrown off more easily.

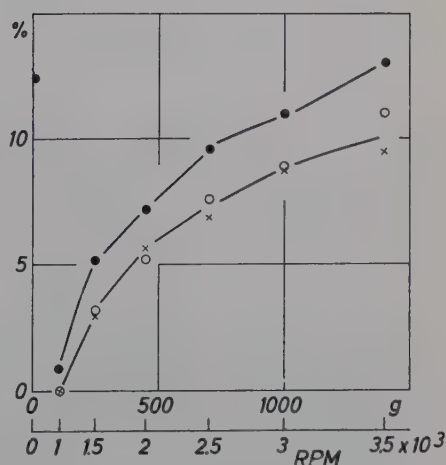
3. Solution loss compared with A.F.S. decrease

In the experiments where the roots were run transversely both standard weight and final weight were determined. Thus it was possible to read the weight decrease of the roots when higher velocities than 1000 R.P.M. were used. The weight decrease (=standard weight — final weight) was determined in the three complete series where A.F.S. was calculated (Figure 1 A) and in two additional series without radioactivity. The results are given in Figure 2 where the means of the given series are shown. An increased amount of solution is removed from the roots with increasing centrifugal force.

To show that there was no irreversible damage or loss in the centrifugation of the roots the series with no radioactivity were run once more after lying for 30 minutes in nutrient solution. This time the velocity was 1000 R.P.M. — the same as in the determination of the standard weight. The roots were found to regain their standard weight except for about 2 per cent. Some of the shorter roots sometimes slipped out of the bundle and were left in the test solution and some root fragments were thrown away in

Figure 2. *Solution loss and A.F.S. decrease of excised wheat roots by transverse centrifugation at different velocities.* On the vertical axis per cent of the standard weight. (=the weight after centrifugation for 30 seconds at 1000 R.P.M. (110 g.). The roots were centrifuged lying at the bottom of a basket 10 cm from the centre. On the horizontal axis the centrifugal force at this distance. The experimental velocities of the centrifuge (R.P.M.) are given on the scale below.

- — the total weight decrease of the roots on the centrifugation (=the standard weight — the final weight).
- — corrected weight decrease=the loss of solution.
- × — A.F.S. decrease.



the centrifugation. The weight decrease has been corrected with 2 per cent of the standard weight in Figure 2.

Further the A.F.S. value for every velocity has been subtracted from the A.F.S. value for 1000 R.P.M. (Figure 2). It should be noted that these A.F.S. values were calculated on the standard weight, so that the decrease of A.F.S. can be compared with the weight decrease for every velocity. The values used in the subtraction differ for this reason somewhat from the values presented in Figure 1 A.

The changes in the A.F.S. values follow completely the weight decrease of the roots caused by solution loss. It is evident that the film which can be removed from the root surface is included in the A.F.S. determined after slow centrifugation. It can be objected that at the highest velocities more than the surface film is thrown off, for instance solution from the cell walls. Therefore the problem was also tackled in a somewhat different way as described in sections 4 and 5.

4. Centrifugation and rinsing

Experiments were performed with wheat roots in bundles in the same manner as in the transverse centrifugations (experiment 1) velocities in the range 1000—3500 R.P.M. being used. Immediately after the centrifugation every root bundle was rinsed in 200 ml distilled water in a beaker. Varying times from 5 to 60 seconds were used. The bundle was moved down and up once every 5 seconds of the rinsing time. Finally the bundle was wiped off twice against the beaker and then transferred to another beaker containing 5 ml 0.1 M MgSO_4 . The roots were then burnt and analyzed according to the standard method. The rinsing water was analyzed for sulphate too. Thus it was determined how many per cent units of A.F.S. were left in the roots after the rinsing and how many were in the rinsing water when the roots had been previously centrifuged at varying R.P.M. The results are given in Figure 3. The value of the A.F.S. left in the roots was practically constant for a given rinsing time; the values after centrifugation at different velocities followed no strict order, thus the mean value is given in the figure. The values of the roots centrifuged at 1000 R.P.M. were on the average 1 to 2 per cent units higher than the values of the roots centrifuged at any other velocity.

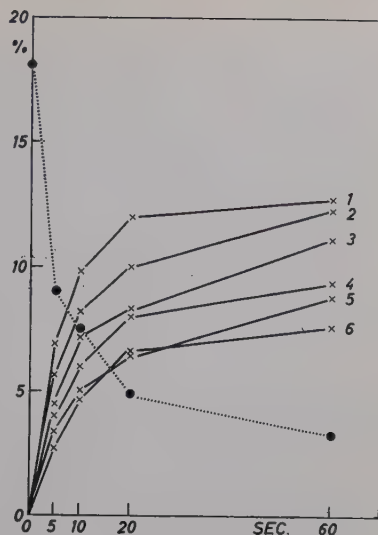
The amount of sulphate left in the rinsing water decreased with increasing velocity of the centrifugation strictly in the order 1000—3500 R.P.M. The difference between the values at the highest and lowest velocities was always about 5 per cent units.

We assume that there is a surface film on the roots. If roots with or

Figure 3. *The effect of rinsing in distilled water on the A.F.S. of wheat roots, centrifuged at different velocities.* Experiments performed according to the standard method at transverse centrifugation. Immediately after the final centrifugation the roots were rinsed for some time in distilled water. The centrifugation velocities in R.P.M. were: 1. 1000, 2. 1500, 3. 2000, 4. 2500, 5. 3000, 6. 3500. The amounts of ions left in the free space of the roots and in the rinsing water were determined. On the vertical axis per cent of the standard weight (=the weight after centrifugation for 0.5 minutes at 1000 R.P.M.). On the horizontal axis rinsing time in seconds.

● --- A.F.S. left in the roots (average of the values for all six velocities). The value for zero time corresponds to 1000 R.P.M. With higher velocities the values decreased down to 11 % for 3500 R.P.M.

× — amount of ions in the rinsing water.



without a surface film are rinsed with water it can be expected that the amounts of ions left in the roots will be about the same but the amounts in the rinsing water will show a difference corresponding to the surface film. This difference should appear already after short rinsing times, say about the diffusion half-time. If the rinsing time greatly exceeds the half-time the difference will show whether it is due to a surface film or not.

The difference obtained in these experiments is thus most likely explained by a surface film. Correction of the A.F.S. value after centrifugation at 1000 R.P.M. with this difference gives an A.F.S. of 13.8 per cent of the fresh weight.

The amount that is washed out during varying rinsing times can be roughly calculated using the corrected A.F.S.-value 13.8 per cent and the amounts of ions left in the free space of the roots (Figure 3).

The following figures are obtained:

Time of rinsing sec.	Per cent of corrected A.F.S. lost by rinsing.
5	36
10	47
20	66
60	77

The ions of the free space are washed out rapidly, the half-time being about 10 seconds.

5. The India Ink method

Bernstein and Nieman (1960) described a method of correcting for the surface film. They used India Ink, (Higgins no 4415) the particles of which should not be able to penetrate the root. It was supposed that the particles of the colloid were not repelled by the root surface.

One experiment has been performed with Higgins India Ink, no 4415. Root bundles were dipped for 30 seconds in a nutrient solution (strength $1/10$ of the standard composition) containing India Ink in the dilution $1/25$ and then taken up and centrifuged transversely according to the standard method. Then the root bundle was transferred to a beaker with distilled water and rinsed for about 30 seconds. The water was coloured black by India Ink carried over on the roots.

The optical density of the rinsing solution as well as of a number of dilutions of the test solution was determined in a Beckman DU spectrophotometer. An amount of the test solution corresponding to 5.6 per cent of the standard weight of the roots was carried on the root surface. The amount approached zero (0.8 per cent) at the highest velocity, 3500 R.P.M.

The mean value of all A.F.S. determinations after transverse centrifugation at 1000 R.P.M., or a centrifugal force of 110 g was 18.5 per cent of the standard weight. Correction with 5.6 per cent as was found with the India Ink method gave an A.F.S.-value of 13.7 per cent of the fresh weight.

The value is essentially the same as that obtained in sections 1—3 and exactly the same as that in section 4.

In the centrifugations at higher velocities a few per cent units may be thrown off from inner parts of the roots. On the other hand, if there is any repulsion of the India Ink particles, the value obtained with this method will be too high. *A.F.S. of excised wheat roots of the age used in these experiments seems to lie within the limits 11—14 per cent.*

Discussion

In A.F.S. determinations the concentration of the medium surrounding the roots is changed. Either the rapid initial uptake is measured or the diffusion out of the root. In both cases the removal of excess solution from the root surface is an essential problem. (Levitt 1957). This removal has been performed by self-drainage, slow centrifugation, or by blotting the roots with filter paper or similar material. The last method is most frequently used. The roots have sometimes been rinsed with distilled water or some solution. The diffusion process is very rapid. Pettersson (1960) reported that the

uptake of sulphate ions in the free space of young sunflower roots was completed within two minutes and leakage experiments with the same material showed that the free space was emptied in about the same time. Mac Robbie and Dainty (1958) calculated that the half-time for K^+ -exchange with "free-space" ions was about 1 second in *Nitellopsis*. The half-times for exodiffusion were calculated by Bernstein and Nieman (1960) to approximately 2 seconds for the roots of bean, barley, wheat and cotton, 12 seconds for pea and 25 seconds for corn. The short half-time for the exodiffusion is striking in our material too. We found a half-time of about 10 seconds for wheat roots.

After a five second rinsing of a root bundle previously centrifuged at 1000 R.P.M., the amount of ions left corresponded to an A.F.S. of only 9 per cent. This short rinsing was more effective than a 30 second centrifugation at the highest velocities used. This experiment does not agree with the results of Jacobson *et al.* (1958). They used phosphate which was not easily removed by rinsing. On the other hand, their assumption that any possible surface film should be removed by a 10 second rinsing is well verified.

The experimental results presented here support Levitt's idea that a surface film on the tissue is included in the A.F.S. when determined with the usual methods. *After centrifugation at 1400 g, A.F.S. of excised wheat roots was determined to 11 per cent. When the centrifugation was combined with the India Ink method as described by Bernstein and Nieman a value of 14 per cent was obtained. The corresponding value after blotting with filter paper was about 18 per cent.*

A.F.S. of excised wheat roots has earlier been determined to 24 per cent by Butler (1953) and to 18 per cent by Kylin and Hylmö (1957). Butler centrifuged the roots in some experiments but worked with low forces, about 50 g. Levitt (1957) recalculated Butler's values and postulated a corrected A.F.S. of 10 per cent in wheat roots, which agrees surprisingly well with the 11–14 per cent obtained in our experiments. Butler (1959) has in later experiments working with sulphate and phosphate ions determined the A.F.S. of wheat roots to 18–20 per cent after blotting with filter paper as Kylin and Hylmö had done. Yet in some experiments the phosphate values were higher.

The shape of the curves in the figures 1 A and 1 B shows that no further reduction of the A.F.S. can be expected if the centrifugal force is increased still more. The excess water seems to be thrown off already at 1000 g, when the mean value of all determinations after transverse centrifugation (experiments 1 and 4) was 12.5 per cent. It can not be mere chance that this force is of the same magnitude as the one used in the determination of the moisture

equivalent in soil. The moisture equivalent is determined by 30 minute centrifugation at 1000 g and corresponds approximately to the field capacity. (Piper 1944). This means that the roots are not unnaturally severely dried when the A.F.S. value is 11—14 per cent. On the contrary, it corresponds to the normal maximal moisture of the roots in nature when the excess water has run off after rain. The step to the permanent wilting point is great (from pF about 2.8 to pF about 4.2; Piper 1944). We can postulate that the root is dried to such an extent by a centrifugation at 1000 g that the water left is attached to the root surface only by adsorptive forces — if the probably hydrophobic areas between the micro-pores are not entirely free from liquid.

Blotting with filter paper corresponds to a transverse centrifugation with a force of 100—250 g in these experiments. At least the greater part of the liquid that is thrown off at higher forces is evidently a film of nutrient solution covering the root surface. It can be calculated that the roots after blotting still have a film 6 to 9 μ thick, if it is uniformly distributed. Levitt (1957) postulated a surface film of 20 μ , using Butler's higher A.F.S. value obtained after a slow centrifugation (50 g) for his calculations. There will, of course, be a great variation in the thickness of the surface film, depending on the efficiency of the method that is used for removing the excess solution.

In the calculation above no account is taken of the root hairs. According to Burström (1957) the total surface of young wheat roots (root-hairs included) can be about three times as large as the area of the root cylinder. *We assume that the solution is uniformly distributed on the root surface including the root hairs. The surface film which can be thrown off from wheat roots rapidly blotted with filter paper is then 2 to 3 μ thick.*

Dainty and Hope (1959) thought that a 5 μ thick film could not possibly have been left on the cell wall surfaces of Chara after blotting with filter paper in their determinations of I.F.S. (iodide free space). Yet a remaining film of about half this magnitude is very likely.

At lower velocities the roots lost more solution when they were centrifuged longitudinally than transversely (Figures 1 A and 1 B). This happened although the greater part of the roots is subjected to somewhat lower forces at longitudinal centrifugation than at transverse when the whole bundle lies at a distance of 10 cm from the centre. The explanation seems to be that the water-holding power is determined by the surface tension, the magnitude of which is fixed by the edge length of the boundary water-root surface. The existence of root hairs makes it difficult to calculate the working forces, but after rough approximations we think that the experimental values of the surface film are of a plausible magnitude.

The thickness of the surface film is likely to have a certain physical

ground. We do not think at present that the thickness decreases laminally at longitudinal centrifugation. Instead we assume that the surface film has a certain thickness and that, dependent on the magnitude of the centrifugal force, the meniscus moves closer to the root tip where the water is dripping away. At transverse centrifugation there are some complications. The roots are packed rather close together which can be expected to increase the water-holding capacity. The edge length is still more difficult to define but it is likely to be longer.

Bernstein and Nieman in a recent paper present a new method of eliminating the surface film error in A.F.S. determinations. They have developed a technique for tagging the external medium with a colloid (India Ink) which does not penetrate the free space. The India Ink particles are not adsorbed to or repelled from the root surface under any of the experimental conditions used. Using chloride ions and mannitol they determined A.F.S. of several species which have earlier been investigated in this respect. The reported values seem to indicate no elimination of the surface film postulated by Levitt (1957). The values are even somewhat higher than those usually obtained with blotting paper. For wheat roots, *e.g.*, they report an A.F.S. of 31.7, 30.6, and 25.0 per cent. These values should be compared with 18 per cent for blotted roots and 11 per cent for roots centrifuged at high velocities in our experiments. However, relatively low values are reported for excised pea roots *viz.*, 8.4, 11.9, 11.6, 7.3, 4.5, 6.6, and 7.2 which may be compared with 7—8 per cent in an earlier investigation (Hylmö 1953) where the surface film should be small since the pea roots had a larger diameter and lacked root hairs.

We used this method in combination with centrifugation (experiments 5) and found that an appreciable amount of test solution was left on the roots after centrifugation at 110 g. The corrected A.F.S. value was 14 per cent of the fresh weight. This value is much smaller than the values obtained for wheat roots by Bernstein and Nieman. It is clear that the variation in the A.F.S. values is highly dependent on the method and the material used for the determination.

One standard experimental series was performed with plants that had grown on nutrient solution for 14 days and thus were of about the same age as the plants used by Bernstein and Nieman. The A.F.S. values fell from 22 per cent at 110 g to 16 per cent at 1400 g. The A.F.S. of these 14-day-old plants was thus 4—5 units higher than that of the 5-day-old plants, used in the experiments presented before. The different age seems to be one reason for the higher values obtained by Bernstein and Nieman.

It may be mentioned that some experiments were made at $4 \pm 1^\circ\text{C}$ to compare A.F.S. in excised roots with that in roots of intact plants. The plants

were put in the refrigerated room 4—5 hours before the experiments were begun. The transpiration from the intact plants was very low. The sulphate concentration used in these experiments was 0.5 mM. No difference was obtained. A trend to higher A.F.S. values in intact plants at high respiration has been reported by Kylin and Hylmö (1957), Bernstein and Nieman (1960) and Pettersson (1961). It may be stressed that the main point in this connection is the difference in A.F.S. of roots from high- and low-transpiring plants. Any difference due only to the fact that the roots are excised or not is rather unlikely.

In the work on the determination of the free space volume of plant roots errors due to a surface film seem to have been eliminated in the investigation reported here. No error or only a small one due to adsorption of sulphate ions or change of the concentration in the Donnan equilibrium is likely. We have tried to reduce the confusion with active uptake processes to a minimum by working at low temperature, 4°C. On the basis of all available evidence it therefore seems safe to conclude that the A.F.S. of wheat roots grown on nutrient solution for 5 days is 11—14 per cent.

Whether this free space available for free diffusion is to be found exclusively in the cell walls or whether the cytoplasm or parts of it are to be included cannot be answered in this investigation.

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Summary

The apparent free space (A.F.S.) of wheat roots grown on nutrient solution for 5 days has been determined with the aid of labelled sulphate ions, the film of nutrient solution adhering to the root surface at first being removed by centrifugation at different velocities. While A.F.S. determined in the usual manner after blotting with filter paper is about 18 per cent the value decreased to 11 per cent after centrifugation. When the centrifugation was combined with the India Ink method, described by Bernstein and Nieman (1960), or a brief rinsing in distilled water a corrected A.F.S. value of 14 per cent was obtained.

As consideration is taken of known sources of error the free space available to free diffusion from the medium is regarded to be 11 to 14 per cent of the volume of excised roots at this age.

In an experiment with 14-day-old plants the A.F.S. was found to be 4 to 5 units higher than that of 5-day-old plants.

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Oxidation of Indole-3-acetic Acid by Fungal Laccase

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The destruction of indole-3-acetic acid (IAA) by enzymes from various plant tissues has been extensively studied (for references, see Ray 1958). In most cases it seems to be due to the action of peroxidases. However, the reaction does not necessarily require hydrogen peroxide, and it is dependent on oxygen. It is usually activated by Mn^{2+} and by monophenols, whereas diphenols like catechol and hydroquinone are inhibitory. These results have been confirmed in experiments with the pure horse radish peroxidase (5) and turnip peroxidase (15).

In fungi, there are several systems with IAA oxidizing capacity. The enzyme from *Omphalia flavida* studied in detail by Ray (11) is a peroxidase with the same general properties as the preparations referred to above. Tyrosinase from the common cultivated mushroom, *Psalliota campestris*, has also been reported to oxidize IAA (1). In this case an addition of catechol or pyrogallol was required, and the suggested mechanism is an oxidation of IAA via an intermediary quinone formed by oxidation of the phenol. Finally, there is evidence that laccase, which is produced by many basidiomycetes (4), is able to oxidize IAA. Tonhazy and Pelczar (13) studied an extracellular oxidase with this property from cultures of *Polyporus versicolor*. The enzyme was not identified, but their data suggest that the enzyme was laccase, the only extracellular oxidase found in this fungus (8). Tonhazy and Pelczar noted that Mn stimulated the oxidation but that 2,4-dichlorophenol was inhibitory. Fahraeus and Tullander (3) studying the induced formation of laccase in cultures of fungi found that laccase-containing culture filtrates readily oxidized IAA. Legrand (7) working with an extract

from some *Lactarius* species, probably containing laccase, observed an oxidation of IAA that was stimulated by diphenols.

The above-mentioned results were obtained with crude solutions, and it seemed desirable to confirm the results by using a highly purified enzyme. Recently such a preparation from *Polyporus versicolor* has been obtained (4, 9), and we have now made a few additional experiments on the oxidation of IAA and some related substances. A more detailed study of the mechanism and products of oxidation was beyond the scope of the present investigation.

The action of the purified laccase on a number of phenolic compounds has been reported in a separate article (2).

Materials and Methods

The laccase preparation was obtained by the procedure described previously (9). Oxidation of IAA was followed manometrically in the conventional Warburg apparatus. The chemicals used were indole-3-acetic acid Merck purum, indole-3-propionic acid (m.p. 110.8—111.5° synthesized preparation, obtained by the courtesy of Dr. Magnus Matell, Uppsala), indole-3-butyric acid Merck, 2,4-dichlorophenol (m.p. 40—41°), catechol Merck p.a., Bacto gelatin Difco, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ Baker p.a. The acids were dissolved in equivalent amounts of sodium hydroxide. In the manometric experiments the following quantities were used: 1 ml 0.1 *M* acetate buffer pH 5.0 (in pH experiment Mc Ilvaine's citrate-phosphate buffer), ca 50 μg . of laccase, 10—20 μM substrate, 0.2 ml. 10 % KOH (in center well) and distilled water to a total volume of 2.4 ml. per vessel. Experiments were run at 25°C.

Experiments and Results

The oxidation of IAA alone and in the presence of dichlorophenol (DCP), Mn ions and catechol was first studied. Since we had previously found (2) that some monophenols inactivated the purified laccase preparations unless protective substances like gelatin or Tween 80 were present, gelatin was also added in some series. The result of this experiment is illustrated in Figure 1.

From Figure 1 the following conclusions can be drawn.

- a) IAA is oxidized directly by laccase also in the absence of any added activators. There is a total uptake of one mole of oxygen per mole of IAA,
- b) the oxidation rate is increased by DCP in the presence of gelatin,
- c) in the absence of gelatin, the effect of DCP is changed into nearly complete inhibition,
- d) Mn has no effect on the oxidation, either in the presence or absence of DCP,

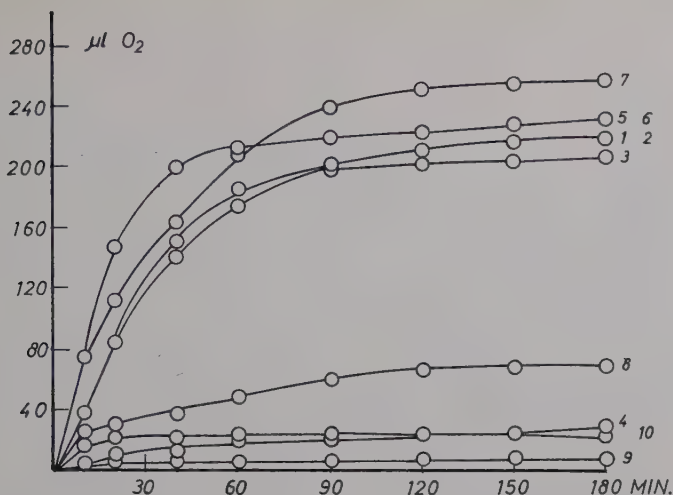


Figure 1. The effect of Mn^{2+} , gelatin, DCP, and catechol on IAA oxidation by laccase: (1) IAA alone, (2) IAA + Mn, (3) IAA + gelatin, (4) IAA + DCP, (5) IAA + gelatin + DCP, (6) IAA + Mn + gelatin + DCP, (7) IAA + catechol, (8) catechol alone, (9) DCP alone, (10) gelatin + DCP. Concentrations used: IAA 10 μ moles, $MnSO_4$ 0.1 μ mole, gelatin 2 mg, DCP 4 μ moles, catechol 2 μ moles, reaction volume 2.2 ml.

e) the oxidation rate is somewhat increased by catechol (compare discussion).

An additional experiment with higher Mn concentration (4×10^{-3} M) showed that this only had a slightly depressing effect on the IAA oxidation rate. $MnSO_4$ alone is not oxidized by laccase.

A pH experiment with values ranging from 3.0 to 6.0 showed that the highest initial velocity was at a pH about 4.0, which is in agreement with results previously obtained with catechol, hydroquinone, and guaiacol (2). At pH 3–4 a certain inactivation of the laccase solutions takes place, and only at pH about 5.0 the reaction goes to completion.

Like some previously studied systems (13, 14), the fungal laccase also oxidized indole-3-propionic acid and indole-3-butyric acid. This is borne out by Figure 2. From the same Figure it is evident that for one mole of IAA oxidized there is an output of one mole of CO_2 . This means that the stoichiometry of the reaction is the same as that observed with other IAA-oxidizing systems. The oxidation products have not been studied by us and, as a matter of fact, even in the case of the extensively studied peroxidase-catalyzed oxidation, the sequence of reactions is not fully understood (Ray 10).

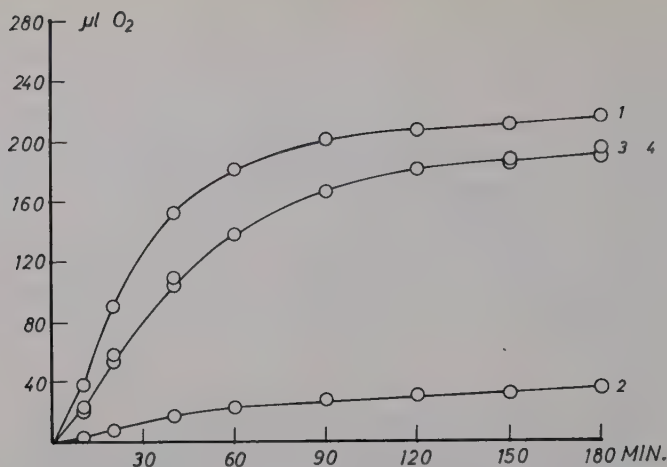


Figure 2. Oxidation of indole-3-acetic acid, indole-3-propionic acid, and indole-3-butyric acid by laccase: (1) IAA in the presence of KOH (for CO₂ absorption), (2) IAA in the absence of KOH, (3) indole-propionic acid, (4) indole-butyric acid. The difference between curves (1) and (2) corresponds closely to 10 μmoles of CO₂ liberated during the reaction.

Discussion

In the laccase-catalyzed oxidation of IAA there is no requirement for additional factors (Mn, monophenols) which are highly active in the peroxidase-catalyzed reaction. 2,4-dichlorophenol (DCP) increases the oxidation rate but the effect is far less than has been found with peroxidase preparations (12). The strong inhibition of laccase caused by DCP in the absence of gelatin seems to be a common effect of monophenols (2, also unpublished confirmation), and may afford an explanation of the inhibitory effect of DCP obtained by Tonhazy and Pelczar (13). These authors, it is true, employed an impure enzyme solution (dialyzed culture filtrate) which probably contained substances that protect the laccase against inactivation. Their enzyme solution was, however, dialyzed for a very long period of time, and our results indicate that also the crude enzyme may be at least partly inactivated by DCP and other monophenols.

From Figure 1 it would seem that also catechol has a positive influence on the IAA oxidation. This effect might, however, mainly be an additive one, since the laccase is not saturated at the substrate concentrations used, and the oxygen consumption corresponds to the amount which can be expected in a mixture where both catechol and IAA are oxidized. In any case the catechol is not inhibitory, a fact which clearly distinguishes this reaction

from that catalyzed by peroxidase. In the latter reaction the inhibition might be due to exhaustion of H_2O_2 ; an addition of this substance brings about a rapid oxidation of the diphenol. With laccase there is no inhibition caused by diphenols, since these will be directly oxidized in the presence of oxygen. No hydrogen peroxide is needed in this case.

As the system laccase-oxygen is largely independent of activators it is in fact the simplest biological system so far known to oxidize IAA. If it has any significance in this respect under natural conditions is, however, completely unknown. Laccase is produced by many fungi which attack living trees and it could possibly in those cases interfere with growth processes in the host plant. But it occurs in purely saprophytic fungi as well, and a definite role of laccase in fungi has not yet been found. It might be engaged in the synthesis and/or breakdown of lignin and lignin-like compounds, but this is still a matter of some controversy (Law 6).

Summary

A study has been made of the oxidation of indole-3-acetic acid (IAA) by a purified laccase from the fungus *Polyporus versicolor*. The results show that IAA is oxidized by this enzyme with the consumption of one mole of oxygen and liberation of one mole of carbon dioxide per mole of IAA. Catechol is not inhibitory, and there is no requirement for Mn^{2+} or monophenols, which differentiates this reaction from the peroxidase-catalyzed reaction studied by several authors. Indole-3-propionic and indole-3-butyric acids are also oxidized by the laccase preparations used, although at a lower rate.

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Glycolytic Activity in Relation to the Growth of *Escherichia coli*

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Introduction

As a preliminary step towards the purification of the enzyme enolase from *Escherichia coli*, it was considered desirable to investigate whether the nutritional conditions of growth of the organism would influence the amount of this enzyme in the cells. Attempts to detect enolase activity in crude extracts or various purified protein fractions from cells grown in peptones or in a synthetic medium with succinate as the source of carbon were unsuccessful. Other conditions of growth were, however, found to give cell extracts with considerable enolase activity.

Variations in the apparent level of an enzyme activity in a crude cell extract may well be due to effects other than differences in the amount of enzyme synthesized per cell. Some of the more pronounced differences in enolase activity presented in this paper are, however, considered to reflect differences in the importance of the glycolytic pathway for the cells. These findings are discussed in relation to differences in the effect of fluoride on the growth under various nutritional conditions.

The results indicate that the glycolytic pathway of metabolism is essential for *E. coli* only under anaerobic conditions of growth with carbohydrates as the sole source of carbon. The synthesis of necessary cellular carbohydrates from organic acids and similar compounds appears to be carried out by other pathways than a complete reversal of the Embden-Meyerhof system.

Material and Methods

Bacterial strains

Most experiments were carried out with a β -galactosidase constitutive strain of *Escherichia coli*, ML 308 obtained from Dr. Melvin Cohn. The results of the growth experiments have, however, been qualitatively controlled with other strains of *E. coli* (K12 and B).

Conditions of growth

Unless otherwise stated, cultures were grown in a synthetic mineral salts medium "56" (9) of the following composition: 0.1 *M* potassium phosphate buffer, pH 7.3; $1.5 \cdot 10^{-2}$ *M*, $(\text{NH}_4)_2\text{SO}_4$; 10^{-3} *M*, MgCl_2 ; $2 \cdot 10^{-5}$ *M*, FeSO_4 . A carbon source was added separately as a sterile solution.

Aerobic conditions were considered to be achieved when only a shallow layer of the culture (not exceeding 1 cm.) had been shaken vigorously in Erlenmeyer or Fernbach flasks on reciprocal or rotary shakers.

Anaerobic conditions were maintained by filling bottles or tubes completely with the culture and then stoppering the containers with glass stoppers.

All the growth experiments were carried out at 37°.

Estimation of bacterial growth

Growth was followed in an Eppendorf electrophotometer using a 405 m μ interference filter. The growth of anaerobic tube cultures was followed directly in the tubes and corrected for differences in the optical quality of the tubes. Samples of the aerobic cultures were transferred to similar tubes before the measurements.

Disintegration of the cells

Cell free extracts were obtained by sonication of cell suspensions, washed twice and suspended in ice-cold 0.02 *M* Tris-HCl buffer, pH 7.3. The apparatus used was the Raytheon 10 kC, 250 W sonic oscillator Model DF101 cooled with circulating ice water. To obtain measurable enolase activities it was usually necessary to concentrate the original cell sample of 50 to 100 ml. to 5 or 10 ml. in connection with the washings. After sonication for 5 min. the extract was centrifuged at $10,000 \times g$ for 10 min. to remove cellular debris. Further sonication was only found to increase the enolase activity of the extract to a small extent.

Estimation of enolase activity

The enzyme activity of the cell free extracts was measured by the optical method of Warburg and Christian (14) and expressed as the initial velocity of the reaction at 25° calculated as the change per min. in absorbancy at 240 m μ . The substrate solution employed had the following composition: $2.4 \cdot 10^{-3}$ *M* DL-2-phosphoglyceric

acid (PGA); $8 \cdot 10^{-3}$ M MgSO_4 ; $2.5 \cdot 10^{-2}$ M sodium phosphate buffer pH 6.8. A Beckman DU spectrophotometer with thermospacers was employed, and the extract was added with a constriction pipet to 1 ml. of substrate solution in a 1 cm. semi-micro quartz cuvette.

When extracts containing relatively high activities were assayed, 25 μl . extract was added and the increase in absorbancy followed with the aid of a potentiometric recorder. Of less active extracts, 100 μl . had to be added, and the absorbancy readings were then usually made without the recorder.

One unit of enzyme activity is defined as that amount which will cause a change of one extinction unit per min. under the described conditions of assay.

Specific activity values are given as units of enzyme activity divided by the protein content of the extract, as estimated by the method of Lowry *et al.* (7). As an example, an extract containing 4 mg. of protein per ml., of which 50 μl . was found to give an absorbancy change of 0.1 per min., is considered to have a specific activity of 0.5.

Although it was found that the enolase activity of an extract was stable at 0° for several hours, the assays were always carried out as soon as possible after the sonication, in order to avoid changes in activity similar to those observed for yeast extracts (v. Hofsten and Tjeder 4).

Results

The enolase activity of E. coli

1. *Conditions of enzyme assay.* — The method employed for the estimation of enolase activity depends upon the formation from 2-phosphoglyceric acid (PGA) of phosphoenolpyruvic acid, which has a strong absorbancy at 240 $\text{m}\mu$. When a suitable amount of an extract of *E. coli* was added to an incubation mixture containing Mg^{++} and a buffer in addition to PGA, a reaction of the type illustrated in Figure 1 was obtained. In the absence of PGA,

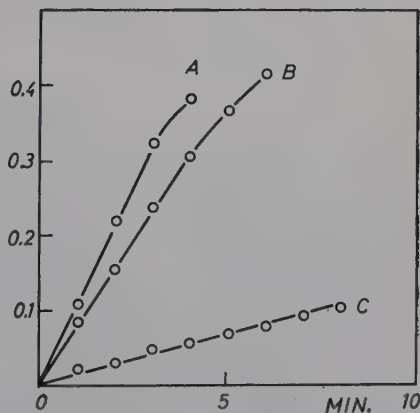
Figure 1. *Time course of the enolase reaction with cell-free extracts of E. coli.* 50 μl of the extract was added to 1 ml. substrate solution under conditions described in the text.

A. Cells from a stationary culture in a glycerol-peptone medium.

B. Cells from a stationary culture in 1 per cent glucose-synthetic medium.

C. Cells from an exponentially growing culture in 0.2 per cent glucose-synthetic medium.

On the ordinate $\Delta E_{240} \text{ m}\mu$.



no endogenous reaction could be demonstrated, and the increase in absorbancy was completely inhibited by 0.01 *M* fluoride and by mercury compounds.

In view of the probable presence in crude cell extracts of many different enzymes that could interfere with the reaction, it is actually quite remarkable that it is possible to detect it at all. As a test for the absence of serious interfering side reactions, a solution of yeast enolase (approximately 80 per cent pure and prepared according to Malmström 8) was added to a bacterial extract. As a result, the increment in the activity measured was found to correspond closely to that expected.

No attempt was made to use optimal conditions for the enzyme assay,

Table 1. *Enolase activity of cell-free extracts of E. coli.*

Cultural conditions	Physiological state of culture at harvest	Units of enzyme activity per mg. protein
<i>Synthetic medium "56"</i>		
<i>Aerobic</i>		
Glucose		
1—2 mg per ml.	Exponential or stationary pH 7	0.09—0.15
10 mg per ml.	Stationary pH 5	0.41—0.44
Succinate		
5 mg per ml.	Exponential pH 7.3	< 0.05
	Stationary pH 7.5	0.05—0.10
Succinate + glucose each at 5 mg per ml.	Exponential pH 7	0.22
Glycerol		
2 mg per ml.	Stationary pH 6.6	0.36
Lactate		
2 mg per ml.	Stationary pH 7.1	0.14
<i>Anaerobic</i>		
Glucose		
10 mg per ml.	Exponential pH 7	0.25—0.26
	Stationary pH 5	0.41
<i>Complex media</i>		
<i>Aerobic</i>		
Difco Casamino acids	Exponential pH 7	< 0.05
Difco Nutrient Broth	Exponential pH 7	< 0.05
	Stationary pH 7.8	0.11—0.18
Nutrient Broth and glucose 2 mg per ml.	Stationary pH 5.5	0.45
Peptone and glycerol 10 ml. per ml. (semianaerobic)	Stationary pH 6	1.04

and only the relative activities of the extracts were considered to be of interest. It is realized that variations in the rate of the reaction measured, may not be true estimates of the enolase content of different types of cells. The data presented are still considered to be of some value for the discussion of differences in the metabolism of cells grown under various conditions.

Variations in the enolase activity of cell extracts. — When *E. coli* was grown under strong aeration in the mineral salts medium "56" with growth-limiting concentrations of glucose (0.1–0.2 per cent), the enolase activity of the cell extract as measured by the technique described was found to be relatively low. If glucose was added at higher concentration, significantly higher activities were obtained especially towards the end of the growth cycle. Similar high values were also found under the anaerobic conditions of growth that are obtained in a stoppered bottle filled to the top with the culture. Table 1 gives the specific enzyme activity values of extracts from cells grown under some varied conditions. The apparent absence of enolase activity in cells grown on succinate or peptone as the source of carbon is particularly noteworthy, but does not necessarily mean that such cells are completely devoid of the enzyme. Some slight activity has also been found in the extract of such cells harvested at the stationary phase of growth.

Good yields of cells and the highest specific enzyme activity so far obtained, 1.04 units per mg. protein in the extract, was obtained from cells grown under semianaerobic conditions (slowly shaken Fernbach flasks) in medium "56" with 1.0 per cent glycerol and 0.1 per cent Difco Tryptone. Purification of the enolase present in extracts from such cells has now been begun, but it has been found that the procedures of acetone and alcohol precipitations commonly used for yeast enolase (Warburg and Christian 14) are unsuitable. Better results have been obtained with ammonium sulfate fractionation and zone electrophoresis, and the results of this work will be published separately.

2. *The effect of fluoride on the growth*

As already reported in an earlier communication (v. Hofsten 3) sodium fluoride has no effect on the growth of *E. coli* if it is added at a concentration of 10^{-2} *M* to aerobic cultures in the mineral salts medium "56" with a carbohydrate, succinate or amino acids, as the source of carbon for growth. In the presence of 0.1 *M* fluoride, the rate of growth is reduced to about half the normal value.

In contrast, the growth of anaerobic cultures of the organism was found to be completely inhibited already by 10^{-3} *M* fluoride, but only if the sole

Table 2. *Inhibitory effect of sodium fluoride on the growth of E. coli.*

<i>Cultural conditions</i> Synthetic medium "56" with the source of carbon indicated	Concentration of NaF (M)	Effect on the rate of growth as compared to a similar culture without fluoride
<i>Aerobic cultures</i> (Shaken flasks)		
Glucose	$2 \cdot 10^{-2}$ or less	No inhibition
Glucose	10^{-1}	50 per cent inhibition
Succinate	10^{-2}	No inhibition
Amino acids	10^{-2}	No inhibition
<i>Anaerobic cultures</i> (Completely filled and stoppered bottles)		
Glucose	10^{-2} — 10^{-3}	Complete inhibition
Glucose+1 per cent glycine	10^{-2}	No inhibition
Glucose+0.1 per cent KNO_3	$2 \cdot 10^{-2}$	No inhibition
Amino acids	10^{-2}	No inhibition

source of carbon was a sugar. The rate of anaerobic growth in medium "56" with glucose is less than one tenth of that of a similar culture grown under aerobic conditions, but the presence of fluoride results in complete cessation of growth. Cultures that have been left more than a week without increased turbidity will, however, immediately begin to grow if oxygen is admitted, which shows that the inhibitory effect is limited to anaerobic conditions. The addition of 0.1 per cent nitrate to the medium increased the rate of anaerobic growth on glucose by a factor of two, and no inhibitory effect of fluoride was obtained in such a medium. Glycine alone cannot be utilized for anaerobic growth, but its presence together with glucose reversed the inhibitory effect of fluoride. Anaerobic growth on succinate, glycerol, or lactate could not be obtained; but on a mixture of amino acids such as the Difco preparation Casamino acids, anaerobic growth is possible, but it is insensitive to fluoride at 10^{-2} M.

Discussion

The fermentative ability of *Escherichia coli* is impressive and the pathways for the degradation of a large number of compounds have been recently reviewed by Rabinowitz (12). It is not the purpose of this paper to discuss the complicated pattern of pathways involved in the early steps of carbohydrate degradation. The relative importance of the glycolytic and oxidative pathways during growth on glucose of *E. coli* has been the object of some controversy and is discussed in detail by Roberts *et al.* (13). The flow of

carbon during synthesis of cellular carbohydrates from Krebs cycle intermediates is also complex, and the changes in enolase activity reported here may indicate differences in the importance of the Embden-Meyerhof pathway in this connection. In the following discussion it is well to keep Axelrod's (1) statement in mind: "It is convenient and proper to talk of pathways but it is often misleading to speak of enzymes belonging to certain pathways".

As already pointed out, great caution must be taken when conclusions are drawn from differences in the rate of an enzymatic reaction carried out with a crude cell extract or whole cells. The estimation of enolase by the increase in absorbancy at 240 m μ may not even be specific, although the complete inhibition of the reaction by 0.01 *M* fluoride would indicate this. The possibility of assaying a purified yeast enolase preparation in the presence of a bacterial extract also shows that the side reactions are relatively less important than might have been expected. No detailed study of the kinetics of the reaction has, however, been made; and an accurate investigation of the biosynthesis of enolase would undoubtedly offer many assay problems. The apparent absence of enolase activity in cells grown aerobically on succinate, lactate, or aminacids as compared with the considerable activity of cells grown on carbohydrates or glycerol, particularly at high concentrations under more or less anaerobic conditions, nevertheless indicate a striking difference in metabolism. The values given for "specific activity" should, however, be taken only as indicative, since the method of protein determination could be subject to criticism in this connection. Other studies by v. Hofsten and Tjeder (4) have shown that the enolase activity of cell extracts of yeast varies in a similar manner.

As a practical consequence of these studies the optimal conditions for the cultivation of *E. coli* in order to prepare enolase, would be to use a medium rich in carbohydrates or glycerol. Completely anaerobic growth would give relatively few cells, and semianaerobic conditions and additions of alkali to neutralize the acids formed have also been found to give better results. The purification of enolase from such cells is now in progress.

The inhibitory effect of fluoride on fermentation was shown by Lohman and Meyerhof (6) to be due specific interference with the enolase reaction, and it was also from fluoride poisoned yeast that Nilsson (11) isolated the accumulating phosphoglyceric acid. Warburg and Christian (14) have further studied the fluoride inhibition of crystalline enolase, and the effect of fluoride on the metabolism of various microorganisms has been reviewed by Werkman and Schlenk (15).

Enolase is thus the classical example of a fluoride-inhibited enzyme. The requirement for phosphate and Mg for strong inhibition indicates the forma-

tion of an enolase-Mg-fluorophosphate complex. No inhibition is obtained in the absence of phosphate, and the substitution of Mg for Zn or Mn will reactivate the enzyme. Other examples of enzymes inhibited by relatively low concentrations of fluoride are carboxylase (Kubowitz and Lüttgens 5) and phosphoglucomutase (Najjar 10) for which the presence of phosphate is not required, and acid phosphate from *E. coli* (v. Hofsten 3), for which there is no indication of a phosphate or Mg requirement. A large number of other enzyme reactions are also inhibited by higher concentrations of fluoride, probably by complex-formation with co-factors or Mg. The reduced rate of growth of *E. coli* in 0.1 M NaF is probably the result of such a general effect.

If a marked difference in sensitivity for fluoride by aerobic and anaerobic cultures of *E. coli* on glucose had not been observed, the absence of inhibitory effect under certain conditions could easily be explained away. It is not easy to calculate the concentration of F^- , when NaF is added to medium "56", and nothing is known about the permeability of the bacterial cell for fluoride. However, other experiments by the author (v. Hofsten 2) have shown that even when it is known that high concentrations of fluoride are produced within the cell, no inhibition of the growth is obtained. Fluoro β -D-galactoside is very effectively transported into the cell by the β -galactoside permease system and then hydrolyzed by intracellular β -galactosidase giving a high concentration of galactose and HF. Galactose-adapted cells of the strain ML 308 were found to be able to utilize fluoro β -D-galactoside as the sole source of carbon for aerobic but not for anaerobic growth. In addition, the absence of inhibitory effect of fluoride on aerobic growth with lactate, succinate, or amino acids as the source of carbon, and the very low enolase activity of cells grown in such media may be taken to indicate that the synthesis of cellular carbohydrates does not depend on the simple reversal of the Embden-Meyerhof pathway.

Conversely, the very marked inhibitory effect of fluoride on the growth of *E. coli* using glucose as the sole source of carbon in the absence of either free or bound oxygen, or when no other suitable hydrogen acceptor is available, may indicate that glycolysis, or more specifically enolase, is necessary for the metabolism.

Summary

1. The enolase activity of cell-free extracts of *Escherichia coli* varies considerably with the conditions of growth. It is minute in cells grown on amino acids or succinate as the source of carbon but high in cells grown on high concentrations of carbohydrates or glycerol.

2. The anaerobic growth of the organism is inhibited by low concentrations of fluoride in a synthetic mineral salts medium with glucose as the sole source of carbon. Similar or higher concentrations of fluoride have no or very slight effect on aerobic growth. Anaerobic growth in the presence of a suitable hydrogen acceptor such as an amino acid or nitrate, is also insensitive to fluoride.

3. The possible significance of these results is discussed in relation to the well-known effect of fluoride on the glycolytic pathway of metabolism. The variations in the enolase activity are considered to indicate that this pathway is indispensable or made extensive use of only under limited conditions of growth.

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The Influence of Boric Acid and Phenylboric Acid on the Root Growth of Bean (*Phaseolus vulgaris*)

By

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Introduction

In a previous paper (Odhnoff 1957) some aspects of the influence of boric acid (B) on root growth were discussed. The physiologic action of B was connected with its property of forming complexes with carbohydrates, and its primary influence was supposed to be exerted on the elongation of the cell wall.

The present discussion on the boron question is generally concentrated on the complex-forming property of the borate ion, although the details of this relationship are still obscure. The work of Caujolle and Bergal (1949 a, b, 1950 a, b) and Torssell (1956, 1957 a, b, c) on the action and chemistry of phenylboric acid (PhB) has provided a new tool in attacking the problem.

The purpose of the present investigation has been to study the influence of B on root growth. To give the problem a new aspect experiments with PhB have been run parallel to B. Some effects on the chemical constitution of the plant have been noted, but the main interest has been focused on the action of B on the cell walls. Its influence is explained together with that of PhB in view of current hypotheses for cell elongation.

Culture Method and Material

The culture method is the same as described earlier (Odhnoff 1957). The nutrient solution was usually changed daily, but it was only half as strong as in the former experiments. B and PhB were added to make the solutions 10^{-5} M when not other-

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wise mentioned. Throughout the paper +B and -B denote presence and absence of B in the nutrient solution, +PhB and -PhB the same for PhB.

As plant material we have used bean (*Phaseolus vulgaris*) of two varieties, Weibull's Stella 41 and Weibull's Alabaster II. Both respond essentially in the same way to boron deficiency, showing macroscopic deficiency symptoms after 3 days in boron-free solution. Their response to PhB was also very similar.

Growth

Morphologic symptoms and dry matter production

The morphologic symptoms of boron deficiency have been described in a previous paper (Odhnoff 1957). The plants acquire thick brittle roots prickly from undeveloped meristems (Figures 1 a, b) at the tips of both the



Figure 1 a. *Eleven-day-old plants treated with B or PhB.* The leaves of the plants -B-PhB are small and irregular with epinastically rolled-in margins, the apical meristem dead and the epicotyl short and thick. The growth of the roots has stopped and they are prickled by undeveloped lateral meristems (cf. Fig. 1 b). A hook at the end of the laterals is often found in this series. — In the PhB-treated plants the leaves are shiny and buckled. The epicotyls are very short but the apical meristems alive, developing small, healthy leaves. There is no difference between plants -B+PhB and +B+PhB. The main roots and the laterals of the first order are longer, but the laterals of the second order shorter and fewer than in the normal plants +B-PhB.



Figure 1 b. *Detail of 5-day-old root tip —B—PhB.* The lateral meristems are formed just behind the apex, but they never develop beyond the meristematic stage.

main root and the laterals. Just before the growth stops the end of the laterals is often bent in a hook. The meristem of the shoot dies shortly after boron deficiency has been evidenced on the roots. Lateral buds may develop, but they die rather soon (cf. Reed 1947). The cotyledons are usually shed at the end of the first week. The chemical analyses have thus been made to the greatest extent on plants in their autotrophic phase, while measurements of elasticity and plasticity have been made on plants still in the heterotrophic phase.

If PhB is added to the plants, the appearance changes in a very characteristic way, irrespective of whether boron is added or not (Figure 1 a). The roots grow long and thin, but the total length of the stems is shorter than +B—PhB. The leaves have a darker green, more brilliant appearance with a buckled surface (cf. Caujolle and Bergal 1949 b, 1950 b, and Torssell 1956). An experiment was run for 21 days with concentrations of PhB ranging from 10^{-7} M to 10^{-4} M added to plants with and without B. In this experiment the nutrient solution was changed every fifth day. The dry weights of the roots, stems, and leaves are shown in Figure 2.

The lowest concentration of PhB, 10^{-7} M, has no influence on the dry matter production. On the boron-deficient plants the laterals are slightly longer and lack hooks. Between plants to which B was added there was no difference with and without PhB at this low concentration.

After addition of 10^{-6} M PhB the dry weight of the boron-deficient roots increases slightly, while the weights of the leaves and the stems are unaffected. Both with and without B the PhB-treated roots grow longer, however. They have a healthy appearance but the laterals seem to be fewer and slightly shorter. The positive effect is also noticeable on shoots of plants without B, where the meristems of the plants —B—PhB often die at an early

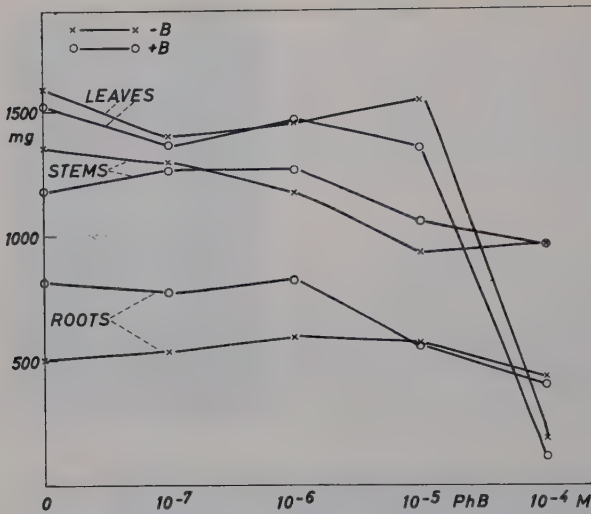


Figure 2. Dry weights of roots, stems, and leaves of sixteen 21-day-old bean plants.

stage, while PhB makes it possible to maintain some growth for a limited time. However, the shoots +PhB are always shorter than those +B-PhB.

In 10^{-5} M PhB the dry weights of the roots +B have fallen to the level of the roots -B (Figure 2). The higher fresh weight of roots -B + 10^{-5} M PhB compared to -B-PhB (Table 1) is statistically significant. The elongation of the roots (Figures 1 a and 4) is stimulated by PhB both with and without B, but they become thinner and weaker. The initiation of laterals is repressed and there are no root hairs. This is a common phenomenon, however, following increased cell elongation (cf. Burström 1950). The dry weights of the leaves +B have in this experiment fallen to about 84 mg. per plant, while the leaves -B have the same weight, 94 mg., as without PhB, indicating a slight PhB-toxication of the leaves in the presence of B not noted without B. This slight toxication is also found on 12-day-old plants while in shorter experiments (Table 1) no difference is noticed. The stems both with and without B weigh about 63 mg. per plant. The length growth of the epicotyl may be slightly promoted compared to -B-PhB, but they are always shorter than +B-PhB.

In $3 \cdot 10^{-5}$ M PhB the plants develop very characteristic toxicity symptoms (Figures 3 a, b). The stem which is hooked only during the first days in normal bean, fails to stretch, becomes thick and cracks in a deep longitudinal fissure at the end of the first week. The toxic limit is obviously lower for shoots than for roots. This is in agreement with the observations of Caujolle and Bergal (1949 b, 1950 b).

PhB in a concentration of 10^{-4} M is toxic to both roots and shoots as well



Figure 3 a. Ten-day-old plants without B. Left without, right with 3.10^{-5} M PhB added. The plumular hook which normally straightens on the 4th to 5th day remains in the PhB-treated plants and the stems grow into spirals.

Figure 3 b. Detail of a hooked stem of a plant treated with 3.10^{-5} M PhB showing fracture into a deep longitudinal fissure and with hyponastically rolled-in leaves.

with as without B. The roots, although growing rather long, are thin and soft and without laterals, the stems become short and thick, and the shoot meristems die.

The significance of the bean hook has been discussed by Withrow *et al.* (1953) and its response to radiant energy and other factors has been extensively investigated by Klein *et al.* (1956) and also by Downs (1955, 1959), all reporting a red-induced straightening of the plumular hook reversed by far-red irradiation. The effect of gibberellic acid (GA) (Lockhart 1958, Figure 2) is apparently not as distinct as the light effect, although the hook seems to have largely disappeared especially in the GA-treated plants grown in darkness. This is very interesting as PhB and to some extent B have an effect opposite to GA also in some other respects, *e.g.*, PhB inhibiting stem elongation and promoting root elongation (Table 1) and GA having the reverse effect especially on the shoot elongation (see *i.a.* Brian and Grove 1957) but also in higher doses on the root elongation (Brian and Hemming 1955).

The lower fresh weight of the roots —B—PhB compared to +B—PhB

Table 1. *Fresh weight in g. per plant, per cent dry matter, and length in cm. of roots, stems, and leaves of 8 to 12-day-old bean plants. Average of 200 plants.*

Treatment	Roots			Stems			Leaves	
	Fresh weight g.	% dry matter	Length (main root) cm.	Fresh weight g.	% dry matter	Length cm.	Fresh weight g.	% dry matter
- B - PhB	0.73	5.6	13.1	1.06	6.3	12.1	0.97	9.2
- B + PhB	0.85	4.4	33.0	0.60	6.9	7.4	0.61	11.9
+ B - PhB	1.27	3.8	18.6	1.08	5.6	22.2	1.07	8.6
+ B + PhB	0.88	4.2	33.0	0.63	7.1	7.9	0.66	11.8

(Table 1) tallies well with the differences in root length (Figure 4) between these two series. The percentage dry matter is somewhat higher in -B-PhB. Only small differences in fresh weight and dry matter are noted between stems and leaves of these treatments. The length of the shoot -PhB increases by 85 per cent, however, on boron addition. A close similarity in both weight, per cent dry matter, and length between plants with PhB and with or without B is obvious in roots as well as stems and leaves. The ratio between length and fresh weight of the roots increases from 1.7 and 1.4 in -B-PhB and +B-PhB respectively to 3.7 in PhB-treated roots with or without B, indicating some fundamental change in the growth pattern upon addition of PhB.

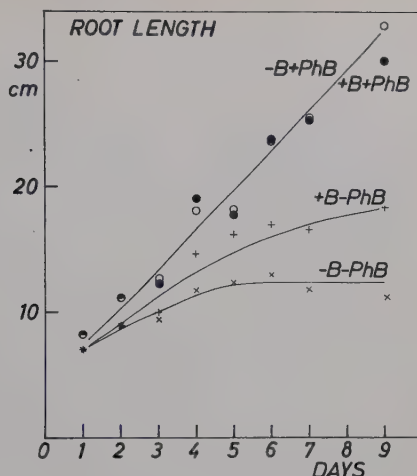
Apparently B has a twofold action on the root growth, both promoting it to a certain extent and simultaneously stabilizing the cell walls, thereby inhibiting excessive elongation. PhB, on the other hand, has only the growth-promoting capacity. Without the stability factor the growth proceeds much longer than in boron-supplied plants without PhB. The growth-promoting factor preponderates over the stability factor, as plants treated with both B and PhB grow in the same way as those -B+PhB.

Root growth

Longer roots can be produced if either the cell divisions become more frequent or the cell elongation is stimulated. To test the action of B and PhB in this respect, we have carried out measurements of the root length (Figure 4) and the cell length (Figure 5) during the first nine and five days respectively, while the different symptoms caused by presence or absence of B or PhB were developing. To obtain a relative measure of the longitudinal cell number, we divided the increase in root length per day with the cell length (Table 2).

The growth rate varies considerably depending on addition of B and PhB.

Figure 4. Action of B and PhB on the growth of the main root.



Roots $-B-PhB$ grow at a slow rate for 4 to 5 days, but then the growth stops. The higher growth rate of the control $+B-PhB$ is usually apparent from the second day. The growth continues during the whole experiment (usually 9 days). The growth of the main root decreases somewhat from about the sixth day, while numerous laterals are developing the whole time. If PhB is added, with or without B the growth rate of the main root is higher than in the control already after the first day, and it continues at a high rate during the whole experiment, correlated with a decreased initiation of lateral meristems, the plants developing the characteristic long thin roots with few laterals previously described. There is no difference in growth rate between PhB-plants with and without B.

Cell length and cell number

The cell lengths (Figure 5) decrease during the experiment in all four cases. A small decrease in cell number (Table 2) is also noted. The values fall into three distinct groups.

1) *Plants $-B-PhB$* have shorter cells than the average already after the first day. The root growth goes on at a steadily diminishing rate, the difference from the normal rate being obvious already from the second day. The cell divisions decrease more rapidly in this group than in the others, the difference being noted on the second to third day.

2) *Plants $+B-PhB$* have already after one day 50 per cent longer cells than the plants without B, but the cell length decreases more rapidly and

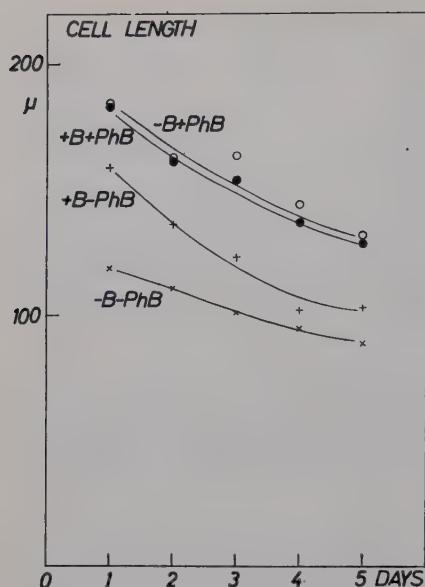


Figure 5. Action of B and PhB on the cell elongation in the main root.

after 5 days they are only 25 per cent longer. The growth rate decreases slightly towards the end of the experiment.

3) Plants $-B+PhB$ and $+B+PhB$. The similarity between these categories is remarkable. Apparently PhB has a very strong growth-regulating effect on the plants, to some extent similar to the effect of B, but with easily recognizable peculiarities. There is no difference in cell length between PhB plants with and without B, but they both have cells longer than $+B-PhB$. The rate of cell division is the same as for plants $+B-PhB$, so the whole increase in root length depends on the increased elongation. Thus PhB has the same action as B in maintaining the activity of meristems once formed, but it changes the growth pattern, enhancing the elongation and repressing the formation of lateral meristems.

Table 2. Relative cell number of the roots (average growth per day divided by cell length). Each figure represents the average of 200 measured plants.

Treatment	Age in days				
	1	2	3	4	5
$-B-PhB$	210	180	149	158	112
$-B+PhB$	216	202	184	194	189
$+B-PhB$	201	199	187	196	185
$+B+PhB$	219	204	195	204	194

Chemical Composition

Soluble sugar

The amount of soluble sugar (total and reducing) was determined according to Philipson (1933) in roots, stems, and leaves of 6 to 7-day-old plants (Table 3). The reducing sugars were considered to be closely equivalent to monosaccharides and the difference between total and reducing sugars, called "non-reducing", was considered to contain the greatest part of the disaccharide fraction.

Plants $-B-PhB$ contain about as much soluble sugar totally as the control $+B-PhB$, in both roots, stems, and leaves. The slightly higher amount in boron-deficient leaves and stems is not statistically significant in the single case, but both values together may indicate some storing of sugar in boron-deficient plants, perhaps depending on some transport difficulty encountered in these plants (Gauch and Dugger 1953). If the transport were blocked, however, a higher proportion of disaccharides at least in the stems would be expected, as sucrose is the common transport form (Mason and Maskell 1934, Ziegler 1956, Zimmermann 1957), but the excess is equally divided between mono- and disaccharides. In the roots there is a slightly lower amount of reducing sugar in boron-deficient plants than in normal ones ($P \sim 0.02$). This may indicate a block already in the conversion of monosaccharides into disaccharides in the leaves and vice versa in the roots (cf. Dugger *et al.* 1957, Scott 1960). The total amount is the same, however, as the boron-deficient plants contain about twice as much non-reducing sugars.

From Whittington's (1959) results it is clear that the sugar status varies with the length of the boron-deficient period. There is evidently no simple relationship between the amount of boron and sugar in the plants. The various results of sugar analyses, indicating either a lower sugar level (White-

Table 3. *Sugar content* (total and reducing as determined on the material, non-reducing as calculated as the difference between total and reducing) in mg. glucose per g. fresh weight of roots, stems, and leaves of 6 to 7-day-old bean plants. Average of 9 experiments. Errors of the mean about 10 per cent.

Treatment	Roots			Stems			Leaves		
	Total	Reducing	Non-red.	Total	Reducing	Non-red.	Total	Reducing	Non-red.
$-B-PhB$	2.59	1.69	0.90	5.68	4.14	1.55	4.68	3.43	1.25
$-B+PhB$	2.47	1.64	0.83	4.94	2.78	2.16	6.03	3.97	2.07
$+B-PhB$	2.75	2.32	0.43	4.94	3.58	1.36	4.01	2.86	1.15
$+B+PhB$	2.05	1.37	0.69	4.72	2.99	1.73	6.36	4.07	2.29

Stevens 1938, Nurmagambetov 1956, Parshikov 1957, Scholz 1959) or a higher level (Baker *et al.* 1956, Odhnoff 1957) in boron-deficiency may thereby be explained. It is hardly probable from our experiments that the sugar content *per se* should cause the growth anomalies observed, as exactly the same values are found in the rapidly elongating PhB roots. Moreover, actual sugar feeding has had very small effect (McIlrath and Palser 1956) or no effect at all (Neales 1960) on boron-deficient plants, neither does boron accelerate the uptake of sugar in plant tissues (O'Kelley 1957, Turnowska-Starck 1959). The transport rate is lower in boron-deficient plants (Zhurbitsky and Vartapetyan 1954, Baker *et al.* 1956), but careful observations show that the disturbance of the transfer of nutrients cannot be noticed until the phloem breaks down (Reed 1947, McIlrath and Palser 1956). The addition of boron does not stimulate the sugar accumulation in sugar cane (Bielleski 1960) although Scott (1960) found a higher amount of sugar in boron-treated sunflowers.

Leaves of plants given PhB — with or without B — contain a significantly higher amount of soluble sugar ($0.01 > P > 0.001$). Both the reducing and the non-reducing fractions are larger, the numerical increase in g. per g. fresh weight being the same.

In roots and stems the total amount of sugar is the same in PhB-plants and normal ones, but a larger fraction is found as disaccharide in root as well as stem and leaves of the PhB-plants. This may depend on an increased rate of conversion of mono- into disaccharides. The reversion of this process cannot be found in the roots, however, as it could be with the possible block caused by boron-deficiency discussed above, the content of non-reducing sugar being higher and reducing sugar being lower than in the control.

Sucrose has been shown generally to increase the elongation of bean hypocotyl sections (Jacobs 1950). Externally added sucrose is probably hydrolyzed, however, on the tissue surface (Burström 1957 a) and taken up as hexose. Therefore, it may be the low level of reducing sugar which limits the elongation of the stems (Table 1) in PhB-treated plants.

Cell wall components

The constituents of the cell walls were analyzed both according to Jermyn and Isherwood (1956) and according to the method used earlier by us (Odhnoff 1957). The residues after every extraction were analyzed for nitrogen and the amount of protein ($N \times 6.25$) per fraction calculated from the differences and subtracted from the data of the fractions.

In both cases the fresh or frozen material was first extracted with boiling ethanol. The residue was considered to contain the total cell wall material. The pectic frac-

tion was then extracted with hot water. According to Jermyn and Isherwood after extraction of pectin the insoluble residue was delignified by treatment with sodium chlorite and the remaining polysaccharides (hemicellulose) extracted with 4-N KOH. No division of the hemicellulose fraction was made, however. The final residue was considered to be cellulose.

According to Odhnoff after extraction of the pectin, the hemicellulose was extracted with 2 per cent H_2SO_4 . The residue was considered to be mainly lignin.

The hemicellulose and the lignin fractions do not interfere with each other in the two methods, however, as the same amount of cellulose is obtained in both cases, provided that correction is made for nitrogen. In the first method about 70 per cent of the nitrogen is extracted together with the lignin and 25 per cent with the pectin, while the extraction with the second method proceeds more stepwise.

The first method gives better reproducibility and is not so strongly influenced by minor temperature fluctuations, *etc.* Only the results obtained with this method are tabulated below (Table 4). The results are calculated on the differences between the fractions and given in per cent ethanol-extracted dry weight.

The lignin and cellulose fractions are very constant, giving an error of the mean between 0.7 and 4 per cent in 6 experiments. The pectin and hemicellulose fractions seem to be less well defined with errors of the mean about 3 per cent in pectin (12 experiments) and up to 10 per cent in hemicellulose (6 experiments). When pectin is low, hemicellulose is high and vice versa. Apparently some component is sometimes extracted into the pectic fraction, sometimes into the hemicellulose fraction.

Boron-deficient plants without PhB contain 16 per cent more lignin ($P < 0.001$) and 9 per cent more cellulose ($0.01 > P > 0.001$). On the other hand, they seem to have less pectin than the control, but the variance is

Table 4. Cell wall substances in per cent of ethanol extracted dry weight from 6 to 8-day-old bean roots. Averages of 12 (pectin) and 6 experiments (protein, lignin, hemicellulose, cellulose), with errors of the mean.

Treatment	Fresh weight per root g.	Ethanol extr. dry weight in % of fresh w.	Protein %	Pectin %	Lignin %	Hemi-cellulose %	Cellulose %
-B-PhB	0.45 \pm .01	5.3	31.1	21.6 \pm .7	19.2 \pm .4	8.5 \pm .9	20.4 \pm .5
-B+PhB	0.74 \pm .02	3.7	32.7	22.8 \pm .6	18.2 \pm .8	6.7 \pm .5	19.3 \pm .1
+B-PhB	0.98 \pm .03	3.8	34.9	22.2 \pm .5	16.6 \pm .2	7.5 \pm .6	18.8 \pm .3
+B+PhB	0.80 \pm .02	3.7	31.7	23.3 \pm .8	17.3 \pm .6	7.1 \pm .6	20.2 \pm .5

Significant differences in fresh weight between all series except -B+PhB and +B+PhB where $P \approx 0.02$. Higher percentage dry weight of plants -B-PhB significant. The difference in lignin and cellulose between -B-PhB and +B-PhB significant at the 0.001 and 0.01 levels.

great and the figures are not statistically significant. Between plants treated with PhB and plants +B—PhB there is no significant difference in any of the fractions analyzed. The high lignin content in plants —B—PhB tallies well with the suggestion of Neales (1960) that lignin formation may actually be enhanced in boron-deficient roots.

Investigating the influence of growth regulators on the composition of the cell wall, Burström (1958) found no quantitative correspondence between the very large growth responses and the small changes in the total amounts of the cell wall fractions. Christiansen and Thimann (1950) and Boroughs and Bonner (1953) working with the effect of IAA on *Avena* coleoptiles and Perlis and Nance (1956) on wheat roots all recorded small changes in the amounts of cell wall material, not nearly proportional to the great simultaneous changes in growth.

Fuller (1958) has measured the change in cell wall polysaccharides in root segments of broad beans of various states of development. Expressed as percentage of the total cell wall polysaccharides measured, the pectic substances decrease only slightly with age. The amount of lignin per cell increased.

The differences between —B—PhB plants and plants +B—PhB as well as those +PhB may simply be interpreted as secondary effects depending on the growth inhibition. As long as growth proceeds at not too low a rate the cell wall is built up from the same constituents in the same proportions independently of variations in the growth of up to 100 per cent. When the elongation stops at an early stage, however, and secondary growth begins earlier than in the control, the amounts of constituents characteristic for the secondary wall — cellulose and lignin — increase, while the amount of primary wall substances — *e.g.* pectin — decreases.

Carotenoids and chlorophylls

The darker colour of the leaves of the PhB-treated plants has prompted an investigation of carotenoids and chlorophylls.

Carotenoids in dark-grown material were extracted according to a modification of a method by Thompson and Kon as described by Goodwin (1955 p. 308).

About 40 leaves weighing about 3 g. are accurately weighed and thoroughly ground in a glass mortar with 1 g. of washed sea sand and 6 g. of a mixture of equal weights of anhydrous sodium sulphate and alumina. The resulting powder is transferred directly to a column (diam. 1 cm.) made of 2 cm. of the same sodium sulphate alumina mixture with 10 cm. dried powdered sugar on top of it.

According to Thompson and Kon this column should be extracted with 2 per cent acetone in hexane to give 95 per cent of the β -carotene present. The hexane extraction gave rather poorly reproducible data, however. From the plants reared in the

Table 5. *Weight in g. per leaf of 6 to 7-day-old primary leaves of bean plants grown with and without PhB in the light and the dark. The values are the average of about 90 leaves in the light and 320 in the dark.*

Treatment	Light	Dark
+ B - PhB	0.47	0.059
+ B + PhB	0.44	0.078

dark we could not extract even a trace of yellow pigment with the above-mentioned hexane solution.

We did not study why this was impossible, but probably the pigments are bound in different ways in light and dark (cf. Burström 1961).

The reproducibility increased if we extracted the chlorophyll-free column with acetone, transferred the pigments into ether and washed 3 times with water. The ether extract was then made up to volume and carotene determined in a Beckman spectrophotometer, λ 440 m μ . The amount of β -carotene present could be calculated from the equation given by Goodwin. The extinction coefficient for β -carotene in ethyl ether=2080 (Kar, as cited by Goodwin p. 296).

Chlorophyll *a* and *b* were determined spectrophotometrically according to Comar and Zscheile (method described by Smith and Benitez 1955 p. 158).

A portion of the acetone extract thus obtained was saponified and the carotene transferred to ether and measured in the same way as the extracts from chlorophyll-free leaves.

The development in light of plants given PhB is described in detail above. The development in the dark differs somewhat from the light pattern apart from the normal dicotyledonous etiolement.

In plants grown in light there is no difference in the weight of the leaves, while the etiolated leaves from PhB-plants grown in the dark have about 30 per cent greater fresh weight than the control (Table 5). If the higher sugar level in PhB plants (Table 3) depends on a more effective mobilization of carbohydrate from the cotyledons, this may be of greater importance when no photosynthesis occurs.

The high amount of sugar in leaves of plants treated with PhB is correlated with a higher content of carotene and chlorophyll (Table 6). The PhB-

Table 6. *Carotene and chlorophyll a and b in mg. per g. fresh weight of 6 to 9-day-old plants. Carotene figures are the average of 13 experiments, chlorophyll figures the average of 10 experiments.*

Treatment	Carotene	Chlorophyll <i>a</i>	Chlorophyll <i>b</i>
+ B - PhB	0.33 \pm .03	1.53 \pm .05	0.60 \pm .02
+ B + PhB	0.36 \pm .03	1.71 \pm .05	0.68 \pm .02

treated plants are generally about 10 to 15 per cent richer in both pigments than the control. The difference in chlorophyll *a* between control and PhB-plants is significant on the 0.02 level, for chlorophyll *b* $0.01 > P > 0.001$.

Carotenoids were determined on light-grown (9 experiments) as well as on dark-grown (4 experiments) plants. There is no difference between these groups, however, and the results have been pooled.

The mean error for the carotene determinations is rather high, however, and these differences are not statistically significant. However, a smaller amount of carotene in PhB-plants is recorded only in 1 of 13 experiments.

Increasing the glucose or sucrose content of the medium of tissue cultures of carrots, Gautheret (1955) found an increased formation of carotene and chlorophyll in the light and of carotene only in the dark.

A similar connexion between the levels of free glucose and of glucosides in wheat shoots was found by Barnell (1936, 1938).

The increase in carotenes and chlorophylls should then be a secondary phenomenon following the high glucose content induced by PhB. That the relationship is not the opposite, *i.e.*, higher chlorophyll content favouring the photosynthesis, is indicated from the results with etiolated plants where no photosynthesis occurs, but still in all 4 experiments a higher carotene level is found. Provided that PhB has a similar effect on the carbohydrate metabolism in light and dark, even the dark-grown PhB-plants should contain more sugar than the control, followed by a greater amount of pigment.

Nitrogen, magnesium, and iron

To test whether the higher chlorophyll content is a secondary effect depending on a possible increase in nitrogen, magnesium, or iron, roots, stems, and leaves were analyzed for total and soluble N and the leaves also for Mg and Fe. Moreover, several authors (see Gauch and Dugger 1954) have presumed a direct influence of B on the nitrogen metabolism.

Table 7. *Nitrogen content of roots, stems, and leaves of 6 to 10-day-old bean plants in per cent dry weight.* Each figure represents the average of triplicate determinations from 7 experiments with errors of the mean of 2–4 per cent for total and 5–10 per cent for soluble nitrogen.

Treatment	Roots		Stems		Leaves	
	Total	Soluble	Total	Soluble	Total	Soluble
– B – PhB	5.8	1.9	6.8	3.8	7.2	1.8
– B + PhB	6.1	2.1	7.0	3.9	7.4	1.7
+ B – PhB	6.0	2.2	6.8	3.7	7.5	1.8
+ B + PhB	6.2	2.1	7.5	4.5	7.3	1.8

Table 8. *Magnesium and iron content of 6 to 9-day-old leaves in mg. per g. dry weight in 3 experiments.*

Expt. no.	- B - PhB		- B + PhB		+ B - PhB		+ B + PhB	
	Mg	Fe	Mg	Fe	Mg	Fe	Mg	Fe
1	3.7	0.14	3.9	0.17	4.4	0.21	3.8	0.15
2	3.5	0.13	2.8	0.12	4.0	0.14	2.8	0.12
3	5.0	0.20	3.5	0.17	4.4	0.23	3.6	0.17

Nitrogen was digested according to the micro-Kjeldahl method, distilled in a modified Parmas-Wagner apparatus into 2 per cent boric acid and titrated with sulphuric acid. Soluble nitrogen was extracted with 2.5 per cent trichloroacetic acid and the extract treated as above.

Magnesium was analyzed titrimetrically according to Scharrer and Mengel (1957). The method was modified to allow determinations of quantities as small as 0.5 mg. Mg.

Iron was determined colorimetrically. The plant material was ashed in an oven at 500°C, but otherwise we followed the method described by Sandell (1944).

All analyses were made in triplicate. There was no difference on the dry weight basis in nitrogen content, total or soluble, between the series with and without B and PhB (Table 7). This is not in agreement with our previous results (1957), where we found a slight increase in nitrogen in the control compared to the boron-deficient plants. Diverging results have been reviewed by Gauch and Dugger (1954). The findings corroborate the assumption that there is no direct connexion between boron and nitrogen metabolism.

Generally the plants -PhB contain slightly more Fe as well as Mg than those +PhB (Table 8). Although the main root is longer in PhB, the root system is weaker (fewer laterals, lower weight) and this may account for the decrease in Fe and Mg. It is puzzling, however, that the leaves nevertheless contain more sugar and also more chlorophyll and carotene than the control.

Elasticity and Plasticity

Theoretical interpretation

We have tested the elasticity of the roots and also made an attempt to determine their possible plasticity. Preston (1955) has elucidated the physical aspects of the problem.

The extensibility of a rod has one elastic and one plastic component. Under a certain stress, it is strained to a corresponding length. At first the strain varies directly with the stress (range *a*, Figure 6), then there usually comes a stage (*b*) during which the strain increases more rapidly than the stress. If the stress at a point P is gradually removed, the body recovers along a

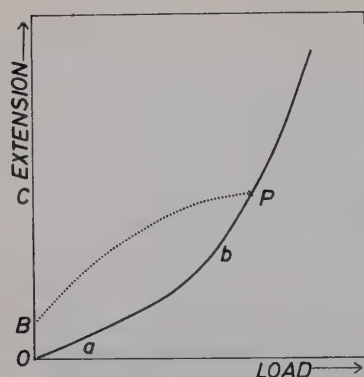


Figure 6. Load/extension curve after Preston and Hepton (1960). For explanation, see text.

curve PB and at zero stress it now has the length B. The part BC of the strain OC may be referred to as the elastic or recoverable component, the part OB as the plastic or non-recoverable component.

The relation between the increase in length (ΔL) of a rod of the length L and the cross-sectional area A , and the load W applied at one end, is called the modulus of elasticity (E), Young's modulus, and may be written

$$E = \frac{W \cdot L}{A \cdot \Delta L} \quad (1)$$

Equation (1) implies a linear relationship between stress and strain and applies therefore only within the region a in Figure 6. Beyond this region, part of the strain is plastic and equation (1) is not strictly applicable (Preston 1955).

With high polymeric substances, such as cell walls, the dimensional change under stress can continue over long periods — the material shows the phenomenon called creep. The importance of these factors, *elasticity*, *plasticity*, and *creep* during the elongation is not clearly understood (Preston and Hepton 1960).

The terms elastic and plastic extensibility are not always sufficient. They signify the ability to undergo a reversible and an irreversible increase in length respectively, while in biologic experiments it is often possible to measure only the actual reversible or irreversible increase in length, the elastic or plastic extension (Heyn 1940). In botanical literature elasticity is often used as a synonym both to elastic extensibility and to elastic extension, the analogue applying to plasticity. We have here distinguished between *plasmometric elasticity* or tensility and *mechanical elasticity*. Plasmometric elasticity indicates the elastic extension as measured as per cent increase in cell length when a tissue is transferred from a plasmolyzing solution to distilled water,

and mechanical elasticity the elastic extensibility as measured by physical methods.

The extension of root tissue has been determined on the epidermis as well as on whole segments from the basal part of the elongation zone by comparing its length in distilled water (or nutrient solution) and in a plasmolytic agent, *e.g.*, mannitol. The turgor pressure is about the same; incipient plasmolysis occurring at about 0.25 to 0.28 *M* mannitol in all series.

A root growing in ordinary nutrient solution may be looked upon as water-saturated and thin epidermis sections do not show any plasticity (Burström 1942). The extension of such single-layer sections should then be only elastic, while in whole root sections, *i.a.*, tissue tension may complicate the results. An attempt to separate possible elastic and plastic components was nevertheless made in the experiments on whole root sections.

The elasticity may be determined by the resonance frequency method, by which the resonance frequency of a tissue in an oscillating magnetic field is measured (Virgin 1955). The method is based upon the fact that a solid body which is caused to vibrate oscillates with a constant frequency characteristic for the body (resonance frequency). A change in rigidity or mass of the body will change its resonance frequency. For the resonance frequency ν , the relation to Young's modulus *E* may be written

$$\nu^2 = K_1 \cdot E, \quad (2)$$

where K_1 is a constant. The method has been further developed and theoretically interpreted by Falk *et al.* (1958) and Nilsson *et al.* (1958). Notes on its practical use may be found in Cleland (1959) and Enckell (1960).

Beside the disparity already indicated by the terms, there is a fundamental difference between mechanical elasticity as measured by the resonance frequency method and plasmometric elasticity as measured as a relation between the length of a turgid and a plasmolyzed cell. When measured by the resonance frequency method, the tissue is only vibrating with an amplitude just wide enough to be perceived in a microscope. The cell wall can be supposed the whole time to keep within the region *a* in the extension load curve in Figure 6, *i.e.*, it is the true elasticity in the physical sense that is measured. With the plasmometric method not only a minimal change in length, just large enough to be seen in a microscope, but the full extension has to be measured (*cf.* Burström 1953, Cleland 1959). This treatment with alternating maximal and minimal extension may in itself induce changes in the quantities to be measured. It must also be remembered that plasmolysis as well as deplasmolysis require some time, in the present experiments as in Burström's about 4 hours, in Cleland's up to 16 hours. Especially in Cleland's experiments some growth must inevitably occur which is then accounted for as

plasticity. Even if the experiments are performed in nitrogen atmosphere, the "creep" component is not accounted for. Furthermore, Heyn (1931) as well as Pohl (1957) have shown that there is a strong decrease in elastic tensility on prolonged treatment in growth-inhibiting media.

Plasmometric elasticity of root epidermis

The extension of the cell wall was measured as per cent increase in cell length when transferred from 0.3 *M* mannitol to distilled water (cf. Burström 1953).

From 20 roots of each series we cut sections from the basal part of the elongation zone, which were put in distilled water, mounted on slides, gently squeezed, and the length of 30 single epidermis cells of each root measured. After that the sections were transferred into 0.3 *M* mannitol, where they became plasmolyzed in a few seconds. Then the length of the plasmolyzed cells was measured in the same way as described above. The difference between the cell length in distilled water and in mannitol, expressed as per cent greater length of turgid compared to plasmolyzed cell, is taken as a measure of the elastic extension.

With regard to their response to the treatment, the plants may be divided into three groups (Table 9).

1) —B—PhB. Already from the second day the elastic extension becomes very small. At this time no effect is shown on the growth or the rate of cell division, but from the first day the cells are shorter than the control. Apparently the decrease in cell elongation is followed by a decreased elasticity. From the third day and onwards the cells are quite unelastic. The growth goes on at a slow rate with decreasing cell elongation and decreasing cell divisions for another day or two.

2) +B—PhB. These are the normal plants, growing fairly well with a slightly diminishing rate for about a week or ten days. The elastic extension

Table 9. *Extension of the epidermis of 1 to 5-day-old roots.* The length of about 30 cells of each root chosen at random from the basal part of the elongation zone, measured first in distilled water (turgid) and then in 0.3 *M* manhitol (plasm.). The extension expressed as per cent increase in length of turgid cell compared to plasmolyzed.

Treatment	Age in days				
	1	2	3	4	5
—B—PhB	9.2	0.9	0.0	—	—
—B+PhB	10.1	10.9	8.7	11.6	9.1
+B—PhB	9.7	7.1	9.8	9.7	3.0
+B+PhB	12.3	9.5	11.6	12.3	7.5

is fairly great at the beginning, but decreases after five days. In the same manner as in $-B-PhB$ the cell elongation decreases before a decrease in elasticity is noted.

3) $-B+PhB$ and $+B+PhB$ behave in the same way. Long roots, long cells, and high values of elastic extension are noticed from the first day. A slight decrease in cell length as well as in elasticity towards the end of the experimental period is found here as in the other groups, the decrease in cell elongation being noted first. The whole time the cells are longer in this group and the elastic extension higher than in the others.

The elasticity may be explained as an effect of the cell elongation which itself is influenced by the growth agent. This is in conformity with Pohl (1953, 1954, 1957), who has shown that the plastic as well as the elastic tensility of an *Avena* cylinder depends on the actual elongation. The results also tally well with those of Preston and Hepton (1960), who found that as the roots grow they become progressively more extensible.

Plasmometric elasticity and plasticity of whole root segments

The plants were either reared in normal solution the whole time or 1 or 2 days before the experiment transferred to 0.025–0.050 *M* mannitol. Some series were grown in 5 \times or 10 \times strength of the normal solution, but the behaviour of these plants was fairly close to the normal ones, and the results have been pooled (Table 10).

The extension was measured on 3 mm. long sections of whole roots, taken from the basal part of the elongation zone. The sections were plasmolyzed for 30 minutes in 0.3 *M* mannitol (A), transferred to distilled water and kept there for 30 minutes (B_1), and then plasmolyzed in 0.3 *M* mannitol again (C_1). This was repeated 2 to 3 times, generally with good agreement between the values for B_1 , B_2 , and B_3 and C_1 , C_2 , and C_3 respectively. The decrease from (B) to (C) denotes the elastic extension and the difference between (C) and (A) the plastic extension. There was sometimes a tendency for the plasmolyzed sections to shrink slowly on prolonged mannitol treatment. The deplasmolysis had then to be prolonged too, and the whole experiment took such a long time that some growth may also have occurred. Therefore the length of the periods was chosen long enough to permit the first fairly rapid reaction to take place, but so short that the results were not contaminated by any growth. In spite of the short experimental period, there was sometimes a tendency of increasing values in moment (B) as well as in (C) when repeated. In Table 10 only the results from the first series of measurements, A , B_1 , and C_1 , are presented.

The plants grown in nutrient solutions of normal, 5 \times , or 10 \times strength

Table 10. Elastic (E), plastic (P), and total extension (Ext) of the basal part of the elongation zone of 3 to 7-day-old roots, measured as per cent change in length when transferred from 0.3 M mannitol to distilled water (Ext=E+P) and to mannitol again (P). Each value is the average of 9–10 experiments with 8–16 plants in each. Error of the mean about 10 per cent.

Treatment	Roots grown in normal, 5x, or 10x concentrated nutrient solution during the whole time			Roots grown in normal solution, but 1–2 days before the expt. 0.025–0.050 M mannitol added		
	E	P	Ext	E	P	Ext
– B – PhB	5.8	3.1	8.9	2.3	0.8	3.1
– B + PhB	9.3	4.1	13.4	4.4	1.2	5.6
+ B – PhB	8.4	3.5	11.9	4.0	1.2	5.2
+ B + PhB	10.4	4.7	15.1	4.5	1.3	5.8

of the normal solution show about twice the elastic and three times the plastic extension of mannitol-grown roots. The lower elasticity and plasticity of the mannitol roots may depend on decreased elongation as discussed in connexion with the extension of epidermis cells. The values for plastic extension are rather uncertain, however. The roots, at least in the weakest nutrient solution, should be water-saturated and not show any plasticity. The results may be explained in connexion with tissue tension or with creep, but neither interpretation seems satisfactory. We have therefore preferred to represent the sum of elastic and plastic extension as total extension which is a quantity measured easily and with good reproducibility.

The relative effects of the growth agents are the same and the results agree well with those from the epidermis measurements.

Mechanical elasticity of whole root segments

The mechanical elasticity of bean roots treated with B and PhB has been determined with the resonance frequency method as described by Virgin (1955) and Falk *et al.* (1958).

The determinations were made on root segments from the basal part of the stretching zone, except on roots –B–PhB, where the length growth of the main root often had stopped and they were developing lateral meristems out to the tips. Here the segment was cut from just behind the tip meristem. The segments were fastened with the basal end in a capillary tube with a diameter just wide enough to let them fit tightly. In the apical end of the root a short spiral (about 1 mm. long) of piano wire was fitted. The diameters of the roots vary with the treatment, those –B–PhB being thicker and those –B+PhB and +B+PhB thinner than the roots +B–PhB (Table 11). The length was chosen to give a resonance frequency preferably

between 60 and 120 p/s, *i.e.*, the segments —B—PhB were about 4 mm. and the others about 3 mm. long as measured from the opening of the capillary tube to the middle of the piano wire.

The elasticity of a tissue depends on the turgor pressure as well as on the elasticity of the cell wall (Falk *et al.* 1958). To measure the last component directly, the tissue must first be plasmolyzed. Therefore the capillary tubes with the root segments were passed through Beckman cuvettes containing 0.1, 0.2, and 0.3 *M* mannitol, and left at least 10 minutes in each. All solutions also contained 10^{-5} *M* K_2SO_4 and 10^{-5} *M* $CaCl_2$. Otherwise the experiments were performed as described by Virgin. Incipient plasmolysis occurred for all series in 0.25 to 0.28 *M* mannitol, and the roots were always flaccid in 0.30 *M* mannitol.

The resonance frequency (ν) has empirically (Falk *et al.* 1958) been found to be directly proportional to the thickness (d) and inversely proportional to the square of the length (l). K_2 being a constant, the formula may be written as

$$\nu = K_2 \cdot \frac{d}{l^2}. \quad (3)$$

The measured resonance frequency (ν) may thus be corrected for differences in d and l by comparing it to a segment of the standard thickness d_c and the standard length l_c , giving the corrected value ν_c of the resonance frequency according to the equation

$$\nu_c = \nu \cdot \frac{\bar{d}_c \cdot l^2}{l_c^2 \cdot d}, \quad (4)$$

where d_c was put equal to 15.0 scale units (1 mm.=22 scale units) and $l_c=5.0$ mm. The results are shown in Table 11.

Due to internal stress always present in plant tissue, a deviation from the ideal value is obtained.

$$E = E_{\text{ideal model}} + E_{\text{internal stress}} \quad (5)$$

Table 11. Resonance frequency (ν_c), relative value of Young's modulus of elasticity (E_{rel}), and diameter of plasmolyzed root segments from the basal part of the elongation zone. 2 to 4-day-old roots. The values for —B—PhB are the average of 100 measurements, the others the average of 50—60 measurements with errors of the mean.

Treatment	ν_c p/s	E_{rel}	Diameter mm.
—B—PhB	$32.2 \pm .8$	1037	$0.86 \pm .01$
—B+PhB	$19.3 \pm .5$	372	$0.70 \pm .01$
+B—PhB	$27.4 \pm .8$	751	$0.75 \pm .01$
+B+PhB	$23.2 \pm .7$	538	$0.69 \pm .01$

(Falk *et al.* 1958, Nilsson *et al.* 1958). By plasmolyzing the roots the factor $E_{\text{internal stress}}$, which must be supposed to be different in roots of different morphology, is kept at a minimum.

The resonance frequency can be measured on roots from all four treatments. It is more difficult to determine on the roots $-B-\text{PhB}$ than on the others, however, depending on their diverging shape — the lateral meristems giving them an irregular larger surface — making the damping effect of the surrounding solution greater on these roots than on the others. In accordance with physical laws for vibration (Barkhausen 1940) the resonance peak of $-B-\text{PhB}$ is therefore much broader than usually obtained.

Young's modulus is derived for an isotropic tissue. It is therefore clear that only tissues with very similar morphology should be compared. There is no visible difference between PhB roots with and without B, either morphologically, or chemically. Soluble sugar, nitrogen, magnesium, iron, cell wall substances of different fractions are closely similar, and so is root length, cell length, and rate of cell division. The difference in elasticity of 50 per cent must therefore be significant and looked upon as a direct boron effect on the cell wall. The comparison of the values for the resonance frequency of $+B-\text{PhB}$ plants with plants given PhB is also good. Morphologically they differ by shorter cells, but this should not influence E (Nilsson *et al.* 1958). In $-B-\text{PhB}$ plants the normal morphology is distorted from the very tip of the root by a great many lateral meristems (Figure 1 b). A quantitative comparison with the other groups is difficult, but the statistically well distinguished ($P < 0.001$) higher resonance frequency in these roots gives strong evidence that the cell walls should be more rigid than those of the others. The highest value of Young's modulus for the rest, *i.e.*, the lowest elasticity, is shown by $+B-\text{PhB}$. Plants $+B+\text{PhB}$ are more elastic and those $-B+\text{PhB}$ most elastic of them all, all differences being fully significant. This is the only difference in any respect found between PhB-plants with and without B. Up till now PhB-plants have behaved in the same way, irrespective of possible boron addition. Chemical constitution, growth, cell elongation, and cell division are very similar, but here is an important difference, giving a *direct proof of the influence of B on the cell wall*.

As pointed out before, the elasticity as measured by the plasmometric method and the elasticity as measured by the resonance frequency method need not be equivalent. Even if the measurements are made on thin epidermis sections reacting practically instantaneously to the osmotic changes, and all interference of plasticity, creep, or growth is ruled out, the plasmometric method gives only the lengthwise extension, while the resonance frequency method gives the true mechanical extensibility. The results obtained by the two methods do not tally completely. The mechanical elasticity has proved a

more sensitive tool in revealing small differences in the structure of the cell wall which may not directly influence the elongation, while the plasmometric elasticity is better correlated with the observed effects on the cell elongation. With single exceptions there is a good agreement between the terms, however, and within limits the plasmometric elasticity may therefore be compared to the mechanical elasticity.

Discussion

During the last years the discussion on the boron question has mainly dealt with the influence of boron on the carbohydrate metabolism. This has been postulated by Schmucker (1934), but those forming a school in this connexion have been Gauch and Dugger (1953, 1954) with the theory that boron facilitates the translocation of carbohydrate in the plant by forming ionized boron-carbohydrate complexes, and they further considered the inhibition of growth in boron deficiency to be caused by sugar deficiency. They have later together and with different coworkers (Sisler *et al.* 1956, Mitchell *et al.* 1960) published further evidence in favour of their original hypotheses.

Dugger *et al.* (1957) found that boron competitively inhibits the action of starch phosphorylase and concluded that the action of boron on the carbohydrate transport is carried out via an effect on the sugar-starch balance. This is also found by Scott (1960) working with B in toxic concentrations.

Some evidence in favour of the sugar translocation hypothesis has been proposed by Chkolnik *et al.* in several publications as reviewed by themselves (1957), Parshikov (1957), and Spurr (1957), but the theories have been doubted at least to be of primary importance by, *i.a.*, McIlrath and Palser (1956), Odhnoff (1957), Skok (1957 a, b, 1958), McIlrath and Skok (1958), Neales (1959 a, b), Turnowska-Starck (1959), and Whittington (1959). Nelson and Gorham (1957) claim their results to be in accordance with Gauch's and Dugger's, but this does not necessarily follow from their data. The present investigation does not show any influence of boron on sugar translocation, neither does a growth inhibition by sugar-deficiency seem likely in boron-deficient plants.

Some connexion of boron with the carbohydrate metabolism seems to be generally recognized during recent years, but then the opinions diverge.

Boron deficiency is often found to decrease cell divisions (Haas and Klotz 1931, Gerretsen and de Hoop 1954, Whittington 1957, 1959, McIlrath and Skok 1958). McIlrath and Skok, however, state that the cell number did not decrease nearly as rapidly as the dry weight with boron deficiency which is also in accordance with our results. Whittington, on the other hand, con-

siders the effect on cell division to be the primary action of boron. In Whittington's latest paper parts of the evidence point in this direction (*e.g.* Figure 2) while, *i.a.*, his Table 1 is contradictory.

Reed (1947) showed that subapical cells were first affected by boron deficiency, while the meristems were still normal. Later Skok (1957 b) from experiments on the influence of boron on the radiosensitivity of sunflower plants concluded that boron primarily affected the cell maturation, not the cell division. Löhnis (1940), Jolivet and Walker (1943), and Walker (1944), found that boron inhibited cell maturation and differentiation more than cell division which could even be enhanced. From the present investigation (Figure 5 and Table 2) it is clear that B as well as PhB have an immediate effect on cell elongation, manifested already the first day, while the effect on cell division does not show up until the second or third day. The main objection to the cell division hypothesis seems to be the observation made, *i.a.*, by Whittington himself, Odhnoff (1957), Neales (1960), and also in the present material (Figure 1 b) that in boron-deficient roots there are a great many abortive lateral meristems formed from within the apical centimetre of the root. After boron addition these meristems grow into normal laterals (Odhnoff 1957, Neales 1960), if they have not been kept too long in the boron-free solution. We therefore consider the inhibition of cell divisions to be a consequence of the inhibited cell elongation. When the cells cannot elongate, the divisions will also stop.

Numerous attempts have been made to substitute other elements or compounds for boron. IAA was not successful (Moinat 1943, MacVicar and Tottingham 1947). Chkolnik *et al.* (1957) found that addition of hydrogen peroxide to boron-deficient plants markedly increased the growth. Germanium which forms complexes with carbohydrates similar to the borate complexes could temporarily substitute B (Skok 1957 a).

PhB is a stable compound (Torssell 1957 a) exerting a specific influence well apart from the boron effect on the process in question, both on boron-deficient and boron-supplied plants. Thorough investigations as to the effect of PhB on a number of dicotyledons have been carried out by Caujolle and Bergal (1949 a, b, 1950 a, b). PhB in low concentrations has no effect on shoots; 10^{-5} M or higher being inhibitory. The root growth, on the other hand, is promoted up to 100 per cent in 10^{-5} M PhB, but stops suddenly at 10^{-4} M. They note that in every species studied the dose for maximal activation of the root growth corresponds with the threshold value for inhibition of the growth of the shoot which is also observed in our experiments. That PhB is not simply a correlative factor, regulating the distribution of some growth hormone, is indicated by its capacity of promoting the growth of roots as well as in some instances of shoots in boron-deficient plants.

Torssell (1956), dealing mainly with arylboric acids from a chemical point of view, gives some data from the effect of PhB on wheat coleoptiles and roots, finding an increase in coleoptile length of about 100 per cent and in root length of 60 to 70 per cent, closely tallying with the increase in cell length.

PhB has no bacteriostatic action (Caujolle *et al.* 1954).

Whether PhB should be called a substituent of B or not is a question of definition. Indeed it may support growth in boron-deficient plants, but in all instances which have been possible for us to analyse the action of PhB may rather be described as an action of a partially disabled boric acid. Haccius (1959, 1960, Haccius and Massfeller 1959) does not share this view, however. In her experiments on the influence of B and PhB on the morphoregulation of plant embryos, she has never found any similarities between the action of B and PhB. B has only shown an unspecific toxic effect or in lower concentrations no effect at all, and never the specific influence on certain developmental processes recorded with PhB. Indeed in the present investigation we have had the advantage of comparing the influence of PhB on boron-deficient as well as boron-supplied plants, but we have also studied a much simpler process and may be inclined to simplify the problem too much. The possibility cannot be excluded that PhB, besides acting as a partially disabled boric acid, also has a specific influence on different growth processes.

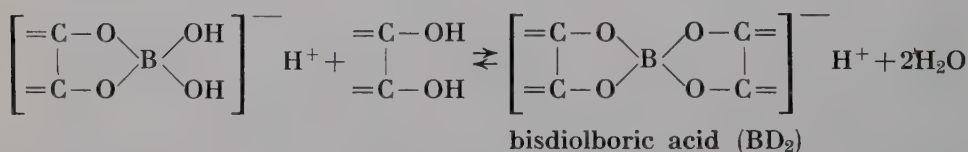
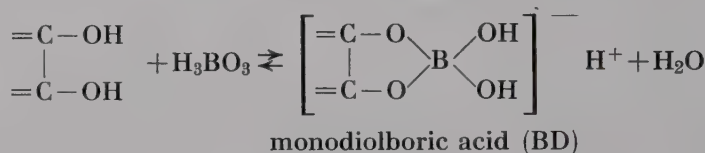
The most remarkable result of sugar, cell wall, and chlorophyll and carotene analyses is the great similarity between plants of widely differing growth patterns in these respects. From Whittington's (1959) results it is clear that the sugar status varies with the length of the boron-deficient period. There is evidently no simple relationship between the amount of B and sugar in the plant. Certainly our results from the cell wall analyses tally well with the findings of both Neales (1960) and Scott (1960), but the simplest explanation seems to be that the change in cell wall composition is a secondary effect brought about by the strong inhibition of the growth in boron-deficient plants. The noticed influence of PhB on chlorophyll and carotene may similarly be explained as secondary effects.

Boron once bound in metabolism is not available for further physiologic utilization (Skok and McIlrath 1958). This is in agreement with Smith (1944) who found that the relative boron content of the cell walls increased with boron deficiency. In a study of the effect of transferring roots from a B-containing to a B-deficient medium Neales (1960) found that radicle tips contained available boron to support the growth for only a few hours. Skok (1957 a) suggests that boron is probably related to a structural unit or "building block" rather than to a metabolic step reaction.

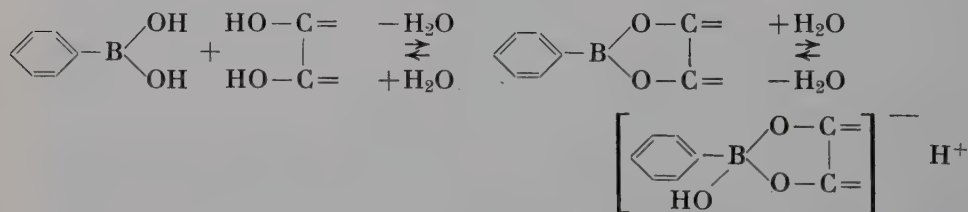
The stubby and brittle roots of boron-deficient plants contrast both to the

normal well-developed control plants and to the long slender roots of PhB-treated plants. Furthermore, Spurr (1957) remarks that celery grown at toxic levels of B was more flexible than normal. It seems, therefore, as if the action of both B and PhB could be traced back to an influence on the rigidity of the cell wall. It is not necessary to presume an action at the same point of the growth, but it is not impossible that PhB and B compete for the same site. In favour of this view is, *i.a.*, the slight toxicity of 10^{-5} M PhB in the presence of B.

Both B and PhB form complexes with polyols. The interaction with boric acid has been formulated by Böeseken (1949):



In PhB one link is already occupied by the phenyl ring and the boric acid rest can only be attached to one sugar residue (Torssell 1957 c).



The BD and BD₂ complexes are formed in different proportions depending on the relative concentrations (Isbell *et al.* 1948, Deuel and Neukom 1949). On biologic material this has been shown by Krejci *et al.* (1949) and it has further been reviewed and interpreted by Zittle (1951). If B is scarce compared to carbohydrate, the complex would be preferably of the BD₂ type, with richer boron supply in relation to carbohydrate, proportionally more BD complexes would form. PhB can only form complexes of the BD type.

BD₂ bonds ought to give a more rigid coupling of the carbohydrate chains than BD bonds which may be supposed to slip more easily over each other, increasing the elasticity of the tissue. In boron-deficient plants without PhB the very small amount of B present should be bound as practically only BD₂.

In normal plants without PhB both BD and BD₂ bonds may be expected. As long as the boron atoms are relatively close in the cell wall, BD complexes will dominate and the elasticity is high. When the distance between the boron atoms increases, relatively more BD₂ complexes are formed and the elasticity decreases. In plants $-B+PhB$ only BD bonds may be formed, the trace of B obtained from the cotyledons being negligible compared to the high amount of PhB; whereas in plants $+B+PhB$ there may exist some BD₂ bonds, but mostly BD bonds. The elasticity should therefore be supposed to decrease in the order $-B+PhB > +B+PhB > +B-PhB > -B-PhB$ which tallies well with our results (Tables 9, 10, and 11).

Boron action should then fit into the picture of the primary cell wall drawn by Preston and Hepton (1960) in order to explain the action of IAA. The BD₂ bridges between hydroxyl groups should be analogous to the Ca-bridges between carboxyl groups between two adjacent molecular chains and the BD complexes comparable to the methyl groups on single chains. The reciprocal effects observed between B and Ca (see *i.a.* Brennan and Shive 1948) and the puzzling interaction between B and IAA (Eaton 1940, Brandenburg 1949) may be interpreted as parallel actions in the same chemical system of B, Ca, and IAA.

Among the current hypotheses for cell elongation there are both those comprising an active growth by intussusception (Ketellapper 1953, Frey-Wyssling 1957, Böhmer 1958, Setterfield and Bayley 1959) and those comprising a passive growth by plastic stretching of the cell wall (Heyn 1940, Cleland and Bonner 1956, Tagawa and Bonner 1957, Cleland 1958, Bonner 1960). According to Ruge (1937, 1942) and Burström (1942, 1957b) the cell elongation involves firstly a loosening of the cell wall and a plastic stretching and secondly an active wall formation by intussusception, the second phase being strongly connected with the carbohydrate metabolism.

Which one of these phases is more likely to be influenced by B and PhB? With the objections as to the interpretations of the plasticity in mind, Table 10 does not exclude an increase in elasticity as well as plasticity upon addition of B or PhB. The connexion between B and PhB and carbohydrate may nevertheless suggest a closer interference with the second phase.

If the extensibility is enhanced, the cell wall may render less resistance to the insertion of new microfibrils. Even if the extensibility of the roots is composed practically only of elasticity, it is suggested by Burström (1954a) and Pohl (1957) and also by Cleland (1959) that elastic extension may be converted into irreversible elongation.

It does not seem probable that the effect of B and PhB on the elasticity should determine the elongation, however. Cell elongation decreases before a decrease in elasticity is noted (Table 9 and Figure 5, cf. also Pohl 1953,

1954, 1957). Also Preston and Hepton (1960) interpret extensibility as a consequence of growth. Moreover, growth may occur to a normal extent in quite rigid coumarin-treated roots (Burström 1954 b).

It seems therefore justified to explain the actions of B and PhB on the extensibility of roots as a consequence of their growth action and not as a cause thereof. The primary growth action may then be connected with the precipitation of new microfibrils constituting the cell walls. Torssell (1956) has shown that the retrogradation or ageing of amylose in water solution is delayed by boric acid. In the cell wall analogous phenomena may occur. PhB may be more effective than B in this respect by forming complexes only with single molecules, while B besides this action also may form bridges between molecular chains, thereby increasing their size and forcing their retrogradation.

This would explain the twofold action of B on the root growth, both promoting it to a certain extent and simultaneously stabilizing the cell walls, thereby inhibiting excessive elongation. PhB, on the other hand, has only the growth-promoting capacity. Without the stability factor the growth may therefore continue longer than in boron-supplied plants without PhB.

Summary

The influence of boric acid (B) and phenylboric acid (PhB) upon the morphology of bean plants has been studied with special interest in the action of the substances on the root growth. A decrease in root length in $-B-PhB$ has been found to depend primarily on a decreased cell elongation, while the cell number is the same in all series during the first days but then decreases in $-B-PhB$. Similarly an increased root length in $-B+PhB$ and $+B+PhB$ compared to the control $+B-PhB$ depends on an increased cell elongation.

The differences in chemical composition (soluble sugars, cell wall components, carotenoids and chlorophylls, N, Mg, and Fe) are small compared to the great morphologic differences and may be explained to a large extent as secondary effects. The present investigation does not show any influence of boron on sugar translocation, neither does a growth inhibition by sugar deficiency seem likely in boron-deficient plants.

The elasticity has been measured with plasmometric as well as with mechanical methods. Within limits the results are comparable. Both methods indicate a low elasticity for roots $-B-PhB$, a medium value for $+B-PhB$ and high elasticity for roots $-B+PhB$ and $+B+PhB$. The plasmometric method does not indicate any difference in elasticity between $-B+PhB$ and $+B+PhB$, while the mechanical elasticity is higher in $-B+PhB$. The results obtained by the plasmometric method tally well with the observed root elon-

gation, while the mechanical elasticity has proved a sensitive tool also in revealing small differences in the cell wall which may not directly influence the elongation. The experimental results indicate that the elasticity of roots is a consequence of their growth and not a cause thereof.

In view of current hypotheses for cell elongation the influence of B and PhB on root growth has been explained as an action on the cell wall probably connected with the precipitation of new microfibrils. A model is proposed, explaining the extensibility of the cell wall as depending on an interaction of B and PhB with carbohydrates, with monodiolboric acids and bisdiolboric acids forming in different amounts depending on the relative proportions of B and PhB compared to carbohydrate.

I wish to express my sincere gratitude to Professor H. Burström, who originally suggested the problem, for helpful discussions, advice, and constructive criticism.

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The Nature of the Germination Inhibitor Present in Leaves of *Eucalyptus rostrata*

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The inhibitory effect of aqueous leaf extracts of *Eucalyptus rostrata* has been demonstrated by Yardeni and Evenari (1952). The aim of the present work was to try to elucidate the nature of the inhibitor and to determine its possible ecological significance on the vegetation under stands of *Eucalyptus*.

Methods

Leaf extraction: Fresh leaves were homogenised in a minimum volume of distilled water. The same water may be used several times by squeezing the suspension through cheese cloth, the liquid then being used to homogenise a fresh quantity of leaves, the final concentration being about 500 g./liter. This suspension was heated to 70°C and kept at this temperature for half an hour in a waterbath. It was then squeezed through several layers of cheese cloth, and the liquid was centrifuged for 20 min. at 2000 r.p.m. The supernatant was used as the crude extract.

Germination tests were carried out with lettuce seeds, variety Progress, as previously described (Lerner *et al.* 1959). Concentration of the test solution is given either as grams of leaves extracted per litre crude extract, or as grams or moles of purified compound per litre.

Results and Discussion

From previous studies (Lerner, Mayer and Evenari 1959), it is known that for the seeds used, the graph for per cent germination plotted against concentration of extract can be divided into three regions:

I. Region of osmotic inhibition; above 6 atmospheres, this region is delimited on its lower concentration boundary by the curve for per cent germination as a function of osmotic pressure.

II. Region of interaction between osmotic and chemical inhibition between 0.5—6.0 atmospheres. When acting simultaneously, the effect of combined, osmotic and chemical, inhibition is greater by a factor of 2—5 than the sum of the effects of both when each is acting separately.

III. Region of chemical inhibition; *i.e.* that region where osmotic effects are negligible, below 0.5 atmospheres.

Inhibitory property of crude extract of Eucalyptus leaves

The crude extract was tested for inhibitory activity by ascertaining the greatest dilution still completely inhibiting germination. The osmotic pressure at this dilution varied from one batch of leaves to another within the range 0.7 atmospheres (about 50 g. leaves per litre) and 2.1 atmospheres (about 250 g. leaves per litre). These results indicate that the inhibition is of the type where chemical and osmotic effects interact (Region II, see above), implying the presence of either large amounts of weak inhibitors or smaller concentration of stronger inhibitors.

Extraction of chemical inhibitor from crude extract

Extraction by ether reduced the inhibitory activity of the crude extract by a half; the ether contained material which inhibits germination completely only at a concentration eight times greater than that required by the original crude extract. Passing the ethereal extract through a column of light-MgCO₃ or alumina, purified considerably the extract while only slightly reducing its inhibitory effect.

In order to produce large amounts of the ether-extractable inhibitor, the crude extract was concentrated in a "flash evaporator" and then extracted with ether. The ether was evaporated and the residue redissolved in a small volume of water and filtered through paper on a Büchner funnel. The inhibitor in the aqueous solution was again extracted into ether and the ethereal solution filtered through an alumina column. The solvent was evaporated, yielding the active material, a yellow oil inhibiting germination completely at a concentration of the order of 400 grams extracted leaves per litre. The oily inhibitory material has the chemical properties shown in table 1. As it is seen from this table:

The Le Rosen and the chloroform-KOH tests for phenol (Feigl p. 135; Pesez and Poirier p. 162) were negative. Moreover the infrared spectrum

Table 1. *Chemical properties of the yellow oil as revealed by spot tests.*

Reagent	Functional group characterized	Reaction	Reference
Sprayed with aqueous solut. of 0.1 % of permanganate	Oxidisable function not specific	Positive; immediate appearance of yellow colour.	Vogel, p. 923 method 1 or 2.
Sprayed with 2,4-dinitrophenyl hydrazine	Carbonyl	Positive; yellow orange colour appears.	Block, Durum, Zweig, p. 344.
Orcinol	Carbonyl	Positive; after heating 5 min. at 100°C, brown colour appears.	
Fuchsin (decolorized)	Aldehyde	Very slow.	
Nitroprusside	Activated methylene group (such as the CH ₂ in α of CO)	Positive; orange ppt. appears in alkaline medium; upon acidification, wine colour appears.	Feigl, p. 223.
1,2-Naphtquinone-4-sulfonate	Reactive CH ₂ and NG ₂	Positive; yellow in alkaline medium changes to brown upon acidification.	Feigl, p. 301.
Vanillin	Probably reactive hydro-gen in α of a carbonyl	Positive; first violet colour appears, changes to black on heating, on further addition of 10 ml of water turns into translucent yellow-pink.	Naves, Papazian, p. 1034.
KOH in alcohol	Not specific	No colour at first, but turns light yellow on heating.	
Diazotized sulfanilic acid NH ₃	Phenols and aromatic amines	Faint rose brown.	Naves, Papazian, p. 1034.
Phloroglucinol	Allyl	Positive; brown.	Massart
Sprayed with aqueous soln. of FeCl ₃ (0.1 %)		Blue black colour appears only after heating a few minutes at 100°C.	Feigl, p. 308.

did not indicate the presence of a phenyl radical, although this is a common functional group of many natural inhibitors.

Tests, according to Feigl, for alcohol, acids and esters were all negative. Molecular weight was estimated by measuring the depression of the melting point of camphor caused by a known amount of the substance. The melting point of a mixture of the inhibitor and camphor gives a cryoscopic depression indicating a molecular weight of the order of 150 to 200. The U.V. spectrum in ethanol showed a wide band with a maximum around 240 m μ ; $\log \epsilon \cong 3$ (molecular weight taken as 200).

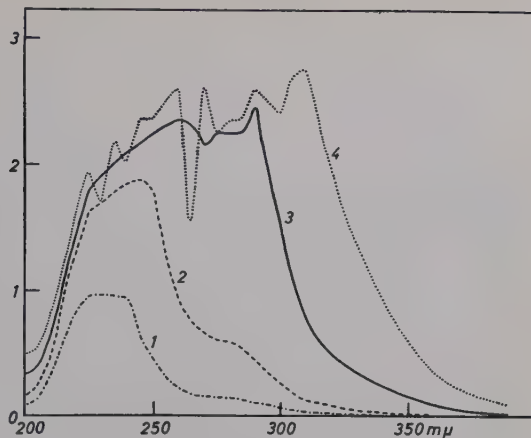
The properties of the inhibitory material as shown by these reactions indicates the presence of both a carbonyl group and a carbon-carbon double bond. The phenyl radical and other functional groups are almost certainly absent. The absorption maximum at 240 m μ . $\log \epsilon \cong 3$, strongly suggests that the carbonyl and the double bond are conjugated (see Cooke and Macbeth 1938).

When larger amounts of the inhibitory material are chromatographed on an alumina column, two yellow bands are observed. The first band-fraction A has a spectrum typical of an unsaturated carbonyl grouping (Figure 1). As it is seen from Figure 1, the absorption is not a linear function of concentration. As concentration increases, the absorption peak broadens towards the visible end of the spectrum and new peaks appear. As is shown in

Figure 2, Mesityl oxide $\left(\begin{array}{c} \text{CH}_3 \\ \text{CH}_3 \end{array} \right) > \text{C} = \text{CH} - \overset{\text{O}}{\parallel} \text{C} - \text{CH}_3$ possesses the same peculiarities in its U.V. spectrum as fraction A, while the spectrum of methyl isobutyl ketone $\left(\begin{array}{c} \text{CH}_3 \\ \text{CH}_3 \end{array} \right) > \text{CH} - \text{CH}_2 - \overset{\text{O}}{\parallel} \text{C} - \text{CH}_3$ shows no such features and its absorption is proportional to the concentration (Figure 2).

After elution of fraction A, the second band was eluted with ether-fraction B. The spectrum of fraction B shows the same properties as that of fraction A. Fraction A was further fractionated into a series of α , β , unsaturated carbonyl compounds by absorbing the oil dissolved in petroleum ether : ether 1/1 V/V on an alumina column. A yellow band-fraction A₁, is hardly absorbed and is rapidly eluted by the same solvent. Further elution with large volumes of this same solvent extracts another α , β , unsaturated derivative — A₂. The column may be further eluted with ether yielding a yellow solution containing A₃, and finally, elution with ethanol yields yet another α , β , unsaturated carbonyl derivative — A₄. No attempt has been made to fractionate fraction B, as it seemed obvious that the inhibitor is a mixture of various essential oils all containing the functional group C=C—C.

Figure 1. Absorption spectrum of fraction A in ethanol at various molar concentrations. 1= $5 \cdot 10^{-4}$, 2= $2 \cdot 10^{-3}$, 3= $1.25 \cdot 10^{-2}$, 4= $5 \cdot 10^{-2}$ M. On the abscissa wave length, on the ordinate optical density.



The absorption spectra, in ethanol, of the five fractions, A₁, A₂, A₃, A₄, and B, at a concentration of 0.1 g./litre (5×10^{-4} molar, MWt being taken as 200) is given in Figure 3; it will be noticed that they all have a maximum absorption peak at 230 mμ. A₂ has a second maximum at 280 mμ; further towards the visible end of the spectrum, its absorption drops sharply to zero, whereas all the other fractions show a very slow decrease in absorption from about 260 mμ to 350 mμ.

Testing the effect of mesityl oxide and isobutyl ketone on germination, it was found that mesityl oxide strongly inhibited germination while the methyl isobutyl ketone was somewhat less inhibitory. It appears, therefore, that the α , β , unsaturated carbonyl group is responsible for the inhibitory action of the yellow oil (see table 2). The concentration of the α , β , unsaturated carbonyl essential oils in *E. rostrata* is of the order of 1 g. oil per kg. of fresh leaves. These derivatives inhibit germination by 50 % at concentrations of the order of 10^{-3} molar, which is equivalent to 0.02 atmospheres *i.e.* well within the region of chemical inhibition where osmotic effects are negligible.

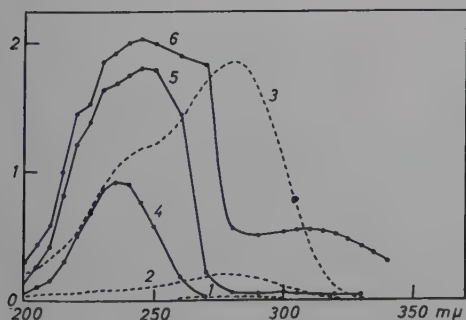


Figure 2. Absorption spectrum of mesityl oxide and methyl isobutyl ketone in ethanol at various concentrations 1, 2, 3 methyl isobutyl ketone, 4, 5, 6 mesityl oxide: 1= 10^{-3} , 2= 10^{-2} , 3= 10^{-1} , 4= 10^{-4} , 5= 10^{-3} , 6= 10^{-2} M. As Figure 1.

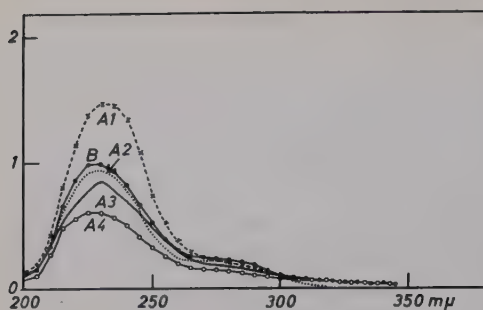


Figure 3. Absorption spectrum of ethanolic solutions of fraction A_1 , A_2 , A_3 , A_4 and B at concentrations of approximately 5×10^{-4} Molar. As Figure 1.

The ether extractable inhibitors having been removed, the aqueous residue of the crude extract inhibits germination completely at osmotic pressures between 1.5 and 4.5 atmospheres which is still within the regions of interactions. Isolation of strong chemical inhibitors of germination from this material has not been successful; inhibition was only apparent at concentrations where the extracts exert considerable osmotic pressure. It is possible, therefore, that very weak chemical inhibitors are present at high concentration and that, where the osmotic effects of the substances are already considerable.

However, fractionation of the aqueous residue of the crude extract did yield a substance which inhibits the development of the radicle. This residue

Table 2. Properties of the α, β -unsaturated carbonyl fractions obtained and of mesityl oxide: maxima of absorption spectrum in ethanol in U.V. log. of extinction coefficient, concentration inhibiting germination by 50 %, fluorescence under U.V. light and odour.

Fraction	λ of absorption maximum in $m\mu$	Log E	Molar concentration at which germination is inhibited by 50 %	Fluorescence under U. V. light	Odour
A_1	232,5	3.5 ¹	$2 \cdot 10^{-4}$	yellow	strong mint
A_2	230	3.3 ¹	$2 \cdot 10^{-3}$	gray	faintly sweet
	280	2.7 ¹			
A_3	230	3.2 ¹	$2 \cdot 10^{-3}$	grayyellow	sweet
A_4	230	3 ¹	$2 \cdot 10^{-3}$	light yellowgreen	sweet reminiscent, of vanilla
B	230	3.3 ¹	$7 \cdot 10^{-4}$	light blue	strong sweet
Mesityl oxide	235	4 ²	$3 \cdot 10^{-4}$	dark spot	
	310	1.7 ³			

¹ at concentration of 0.1 g./litre equivalent to $5 \cdot 10^{-4}$ molar.

² at concentration of 10^{-4} molar.

³ at concentration of 10^{-2} molar.

was filtered through a column of Whatman paper No. 1. Water was then percolated in order to wash away all unabsorbed material. The washed column contained brown material at the top, which could be eluted with 10 % aqueous acetic acid. The latter was dried in a "flash evaporator" (40°C), the residue was then taken up in a small volume of water and again evaporated to dryness. This procedure was repeated until the odour of acetic acid had completely disappeared. The brown precipitate contained two compounds which were separated from one another by their difference in water solubility. A few consecutive extractions of the precipitate with very small quantities of water dissolved the root inhibitor. The rest of the precipitate could be dissolved with more water, but did not inhibit root growth. The root inhibitor had no effect on germination, but the roots of the seedlings failed to develop; it was the hypocotyls that protruded from the seed coat. At high concentration of the inhibitor, a brown spot could be seen at the tip of the hypocotyl and no root developed at all, while at lower concentrations, a brownish root developed without root hairs. Certain batches of leaves contain considerable amounts of this root inhibitor, while other batches contain none at all.

It seemed of interest to determine whether the soil taken from underneath Eucalyptus leaf litter had any effect on germination.

Two groves in the vicinity of Natanya (Israel), were selected. In one grove, the trees grew in light sandy soil, while in the other, soil was heavy and covered with a great deal of partly decomposed leaf litter. The soil was taken from a place situated 5 metres inside each grove; the control samples were taken 5 metres outside each grove, 10 metres away from the previous sample place. The soil was tested for its effect on the germination of lettuce and wheat seeds. The experiments were carried out in pots. All pots, including the ones with the decomposed leaf litter, showed, after a few weeks, almost identical germination and growth. These results indicate that the inhibitors present in the leaves do not accumulate to inhibitory concentrations in the soil beneath the trees but are probably destroyed. It does not appear therefore that the compounds isolated from the leaves are in themselves of ecological importance in controlling the vegetation in Eucalyptus groves.

However, it was shown for the soil coverage under oak trees (Dinoor 1959), that the layer of the decomposed and partly decomposed leaves serves as a sort of a physical barrier for the germinating seedlings. The roots of seeds, germinating on the layer of litter, can not penetrate into the soil while the shoots of seeds, germinating in the soil underneath the layer of litter, can not penetrate through the layer into the air and light. A similar condition may also prevail under the Eucalyptus trees, the small amounts of

inhibitory substances still present in the leaves, may interact with the physical effects in controlling the vegetation underneath these trees.

But more investigation in the problem is needed before this can be said with any certainty.

Summary

The leaves of *Eucalyptus rostrata* contain substances that inhibit growth and germination. The nature of these substances was investigated: The crude extract was fractionated into four fractions, the U.V. spectrum and other properties of these fractions were investigated.

The properties of the inhibitory materials indicate that they contain a carbonyl group and a carbon-carbon double bond. Mesityl oxide was shown to have properties similar to the inhibitory substances from the Eucalyptus leaves.

The soil from beneath the Eucalyptus trees was also investigated. The results suggest that the inhibitors present in the leaves do not accumulate in the soil to inhibitory concentrations. Therefore it does not seem that the inhibitory substances as such, are of ecological importance but together with the physical effects of the leaf litter, they may control the vegetation underneath these trees.

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Effects of 2,3,6-Trichlorobenzoic acid on Klinostatically Developed Geotropic Curvatures of *Avena* Coleoptiles

By

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Introduction

Several recent publications have been concerned with the selective inhibition of negative geotropic curvature of *Avena* coleoptiles and other plant stems (2, 4, 5, 7, 8). Dilute solutions of 2,3,6-trichlorobenzoic acid, 2,6-dichlorobenzoic acid and naphthylphthalamic acid have received major consideration. Vander Beek (8) reported that stems of oats, barley, cucumber, and other seedlings failed to assume the normal vertical position in darkness when grown in 1×10^{-6} to 1×10^{-4} *M* solutions of chlorinated benzoic acids. More recently, Schrank (6) found that certain concentrations of 2,3,6-trichlorobenzoic acid (TCBA) increased elongation of isolated apical coleoptile sections, but inhibited upward bending. These results were interpreted to indicate that TCBA was primarily involved in the inhibition of the geotropic perception mechanism. There also was some indication in these results (Schrank 6) that the TCBA in combination with the endogenous auxin gave a total concentration of growth promoting substances high enough to have a slight masking effect on curvatures in some instances.

The present study is concerned with the effects of TCBA on geotropic curvatures of 15 mm. apical *Avena* sections which were developed on the klinostat subsequent to geotropic stimulation.

Materials and Methods

Avena sativa seeds, Victory Strain (U. S. Department of Agriculture, C. I. 2020 supplied by the U. S. Department of Agriculture) were used in these experiments. The seedlings were grown at 22°C ($\pm 1^\circ$) on filter paper strips immersed in dis-

tilled water which previously had been aerated. Additional details of the growing method have been described elsewhere (9). Only seedlings that were 72 hours old and that had 30 mm. (± 2 mm.) coleoptiles were used.

Fifteen mm. apical coleoptile sections were used in the growth measurements. These sections had intact apices, but the primary leaves were removed. The isolated coleoptiles were mounted on sections of stainless steel needles with stops 5 mm. from one end. The individual coleoptile holders were fitted into lucite blocks as previously described (Schrack 6). Only the cut basal ends of the coleoptiles were exposed to the aerated distilled water or other solution. Lengths of the coleoptiles were measured with a micrometer in the ocular of a dissecting microscope. During cutting and transferring, the sections were exposed to red neon light of low intensity (wave lengths longer than 607 m μ). All growth and curvature experiments were performed in the same red light.

Curvature measurements also were made on apical 15 mm. coleoptile sections, which were mounted in the same holders that were used in the growth experiments. The coleoptiles were left for 2 hours in the holders in the upright position before they were geotropically stimulated. After geotropic exposure and curvature development time, the angles of curvature were measured from negatives which were made by shadowgraphing. In the indicated instances, curvature development or growth was completed on a horizontal klinostat rotating at one revolution per minute. The coleoptile holders, which were to be attached to the klinostat, were covered with a layer of agar 3 mm. deep to prevent the loss of liquid during rotation. Most of the procedures were carried out at 22°C ($\pm 1^\circ$), but in some instances, an experimental temperature of 4°C was utilized.

Results

Previous results (6) have shown that 1×10^{-4} M TCBA increased elongation of 15 mm. apical segments of *Avena* coleoptiles when they were kept in the upright position. As shown by Curves I-V and I-K in Figure 1, rotation on a horizontal klinostat at one revolution per minute for 24 hours did not alter the elongation rate of coleoptiles in the absence of TCBA. Both of these curves were obtained at 22°C. Coleoptiles, which were exposed to 1×10^{-4} M TCBA, revealed a slight increase in growth rate during the first 8 hours of klinostatic development (Curves I-VT and I-KT). While this increase was small, it was consistently observed. The magnitude of the growth increase induced by the TCBA (compare Curves I-K with I-KT) on the klinostat is similar to the extent of the effect on coleoptiles grown in the vertical position.

As indicated by Curves II-V and II-K, at 4°C the coleoptile growth rate was lowered. As before, rotation (Curve II-K) did not influence the growth rate. On the basis of the final lengths attained in 24 hours, the Q_{10} for elongation of apical coleoptile segments was 1.84. This value is essentially the same as obtained by Brauner and Hager (1) for *Helianthus* seedlings.

Curve I-V. Elongation of coleoptiles at 22°C in the vertical position.

Curve I-K. Elongation of coleoptiles at 22°C on the klinostat.

Curve I-VT. Elongation of coleoptiles treated with 1×10^{-4} M TCBA and kept in the vertical position at 22°C.

Curve I-KT. Elongation of coleoptiles treated with 1×10^{-4} M TCBA and rotated on the klinostat at 22°C.

Curve II-V. Elongation of coleoptiles at 4°C in the vertical position.

Curve II-K. Elongation of coleoptiles at 4°C on the klinostat.

Curve II-KT. Elongation of coleoptiles treated with 1×10^{-4} M TCBA and rotated on the klinostat at 4°C.

On the ordinate elongation mm.

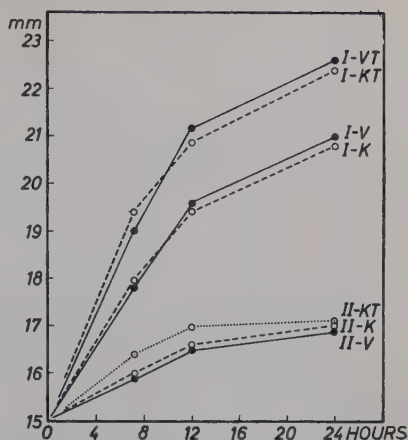


Figure 1. Growth of 15 mm. apical *Avena* coleoptile sections. Each point represents an average of 40 or more measurements.

The effect of 1×10^{-4} M TCBA on elongation on the klinostat at 4°C is shown by Curve II-KT. Under these conditions, TCBA caused a small but significant increase in elongation for 8–12 hours. This stimulating effect was proportionately more extensive at 4°C than at 22°C.

The results in Figure 2 show that magnitude of the curvatures attained after 4.5 hours in the horizontal position is directly dependent on the temperature with a Q_{10} for 4°–22°C equal to 2.0. This appears to indicate that final curvatures reached in this time are limited by the elongation mechanism. After 2 hours in the horizontal position, only 8° of curvature developed at 4°C as compared to 56° at 22°C. These curvatures give a Q_{10} of 2.9. It is also significant to note that the lower temperatures delay the initiation of upward curvature.

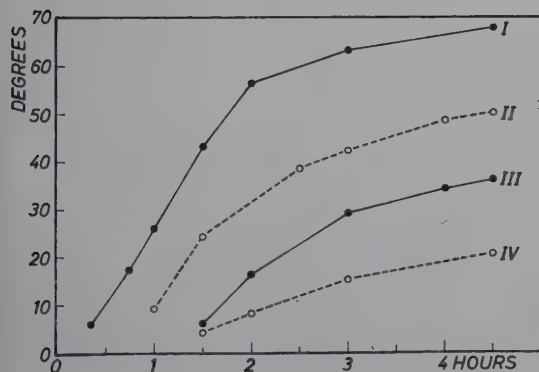


Figure 2. The effect of temperature on negative geotropic curvature of 15 mm. apical *Avena* coleoptile sections. Temperatures are indicated for each curve. Each point represents the average of 40 or more measurements. On the abscissa exposure time, on the ordinate degrees of curvature.

I 22°
II 15°
III 10°
IV 4°

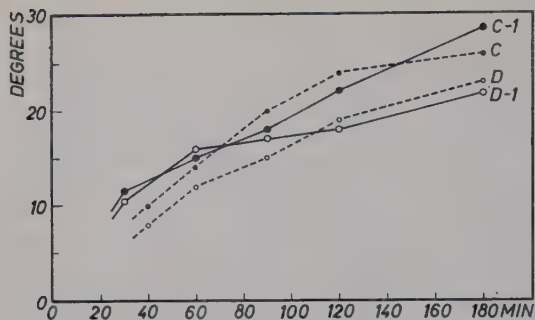


Figure 3. The effect of 1×10^{-4} M TCBA on geotropic curvature of 15 mm. apical *Avena* coleoptiles which were geotropically stimulated at 4°C and developed on the klinostat for 90 minutes. Each point represents an average of 40 or more measurements. As Figure 2.

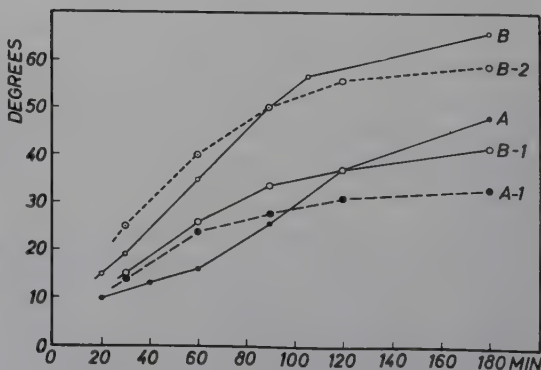
Curve	Klinostat	TCBA
C	4°	0
C-1	4°	10^{-4} M
D	22°	0
D-1	22°	10^{-4} M

As indicated by Curve B in Figure 4, the maximum rate of bending of 0.5° per minute occurred for coleoptiles which were maintained in pre-aerated distilled water when the geotropic stimulation was applied at 22°C with subsequent klinostat development at 4°C for 90 minutes. The rate of bending indicated by the slope of Curve B for the first 90 minutes of exposure is essentially the same as the slope of the corresponding portion of Curve I in Figure 2. This means that the klinostat time at 4°C did not increase the rate of upward bending, but it did allow time for the development of an average of an additional 10° for a given exposure time up to 90 minutes. The results shown by Curve A in Figure 4 are from coleoptiles that were stimulated at 22°C and kept on the klinostat at 22°C . It is evident from these Curves A and B that 90 minutes of klinostat time at 22°C is inhibitory to geotropic bending of coleoptiles.

When the stimulation was applied at 4°C , the temperature during the klinostat development had only a slight effect on geotropic curvature. This is revealed by Curves C and D in Figure 3. Exposures of 180 minutes plus 90 minutes of klinostat time produced an average of about 25° of curvature.

Figure 4. The effect of TCBA on geotropic curvature of 15 mm. apical *Avena* coleoptiles that were geotropically stimulated at 22°C , and developed on the klinostat for 90 minutes. Each point represents an average of 40 or more measurements. As Figure 2.

Curve	Klinostat	TCBA	M
A	22°	0	
A-1	22°	$1 \cdot 10^{-4}$ M	
B	4°	0	
B-1	4°	$1 \cdot 10^{-4}$ M	
B-2	4°	$1 \cdot 10^{-4}$ M	



For shorter geotropic exposures, development on the klinostat at 4°C produced slightly greater curvatures than development at 22°C. Coleoptiles which were kept in the horizontal position at 4°C manifested an average curvature of 15° at the end of 3 hours (Curve IV, Figure 2). As before, the klinostat time mediated the development of an additional 10° of curvature for a given duration of stimulation, but in this instance approximately equal final curvatures developed at 4°C and 22°C on the klinostat.

Results of a previous study (Schrank 6) have demonstrated that 1×10^{-4} M TCBA effectively inhibits negative geotropism of *Avena* coleoptiles when they are kept in the horizontal position. Figure 3 also shows the effects of TCBA on geotropic curvature of coleoptiles which were stimulated by exposure in the horizontal position at 4°C. For geotropic exposure times up to 100 minutes, 1×10^{-4} M TCBA caused an increase in negative bending. (See Curves D and D-1.) This initial increase in curvature very likely was caused by the stimulating effect of TCBA on growth. Previous data have shown (Figure 1) that 1×10^{-4} M TCBA excited elongation of coleoptiles rotating on a klinostat. This was especially significant during the first 12 hours of growth. Longer development of geotropically stimulated coleoptiles on the klinostat at 22°C, as revealed by data in Figure 3, when compared to previously published results (Schrank 6), caused slight inhibition of curvature for durations of stimulation longer than 60 minutes. 1×10^{-4} M TCBA had no effect on elongation of 15 mm. apical coleoptile sections in the vertical position at 4°C. Geotropic curvature, mediated by long exposures in the horizontal position, is inhibited at 4°C by this concentration of TCBA, as indicated by previously published evidence (Schrank 6). The combination of these results indicates that the stimulating effect of TCBA on elongation at 22°C more than counteracts the inhibitory effect of the TCBA on geotropic perception at 4°C.

When coleoptiles were geotropically stimulated and developed at 4°C (Curves C and C-1 in Figure 3) with 1×10^{-4} M, a slight increase in curvature was observed for exposures up to 60 minutes. This increase in curvature apparently is a reflection of the fact that 1×10^{-4} M TCBA caused a small stimulation of elongation at 4°C on the klinostat. (See Curve II-KT in Figure 1.)

As shown by Curves A and A-1 in Figure 4, when coleoptiles were treated with 1×10^{-4} M TCBA and developed on the klinostat at 22°C, upward curvature was stimulated for exposures up to 90 minutes and inhibited for exposures longer than 100 minutes. Since this concentration of TCBA is known to stimulate elongation, (Figure 1) (see also Schrank 6), the stimulation of curvature for short exposures could be caused by the stimulation of elongation by TCBA. The inhibition of curvature by 1×10^{-4} M TCBA for geotropic

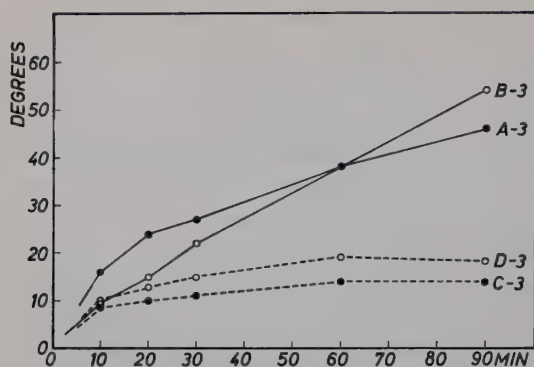


Figure 5. The effects of variation of stimulation temperature and the temperature of klinostatic development for 30 minutes on negative geotropic curvatures of 15 mm. apical *Avena coleoptile* sections. Each point on the curves represents an average of 40 or more measurements. As Figure 2.

Curve	Stimulation	Klinostat
A-3	22°	22°
B-3	22°	4°
C-3	4°	4°
D-3	4°	22°

exposures longer than 100 minutes must be caused by some other effect of TCBA.

For Curves B, B-1 and B-2 in Figure 4, the coleoptiles were developed on the klinostat at 4°C. As indicated by Curve B-1, when compared to Curve B, 1×10^{-4} M TCBA caused about a 30 % curvature inhibition for all the indicated durations of geotropic stimulation. The slight stimulatory effect of 1×10^{-4} M TCBA on elongation is completely masked by the inhibition of geotropic perception by this concentration of TCBA at 22°C. This is consistent with previous results without klinostat development (Schrank 6). When 1×10^{-5} M TCBA was used (Curve B-2), with low temperature klinostat development, increased curvature was observed for exposures up to 90 minutes and inhibition of bending was recorded for longer periods of geotropic exposure. The initial increased curvature again may be caused by the effect of TCBA on elongation, but the subsequent curvature inhibition depends on some additional processes.

The results shown in Figure 5 are from experiments similar to the previous ones except that in this instance the klinostat development time was limited to 30 minutes. It is apparent from the curves in this figure that 10 minutes of exposure in the horizontal position was adequate to initiate bending. Curve B-3 in Figure 5 essentially duplicates the first half of Curve B in Figure 4. Thus when stimulation was applied at 22°C, klinostat development at 4°C for 30 minutes was just as effective as development for 90 minutes at the same temperature.

As previously noted, development on the klinostat at 22°C for 90 minutes inhibits upward curvature (Curve A, Figure 4). For exposures up to 60 minutes, klinostat development for 30 minutes at 22°C results in larger curvatures than similar development at 4°C. This is shown by Curves A-3 and B-3 in Figure 5. As indicated by Curve A in Figure 4 and Curve A-3 in

Figure 6. The effect of 1×10^{-4} M TCBA on geotropic curvature of 15 mm. apical *Avena coleoptiles* that were placed in the horizontal position for various durations and then developed on the klinostat for 30 minutes. Each point represents an average of 40 or more measurements. As Figure 2.

Legends for the curves:

Curve	Stimulation	Klinostat
A-4	22°	22°
B-4	22°	4°
C-4	4°	4°
D-4	4°	22°

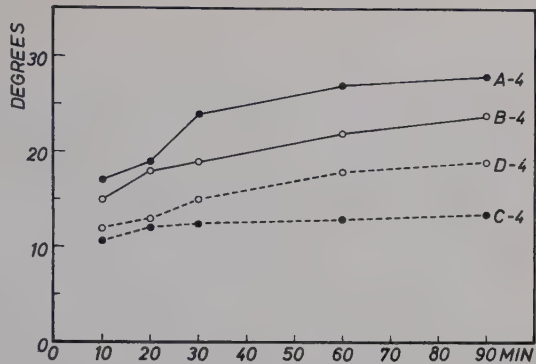


Figure 5, development for 30 minutes at 22°C mediates curvatures more than twice as large as similar development for 90 minutes for stimulation times up to 60 minutes or more. This observation apparently is a substantiation of the fact that long development on the klinostat at 22°C gives the coleoptiles time to partially straighten out before the curvatures are measured. Development on the klinostat for 30 minutes at 22°C results in even greater curvatures than development for 90 minutes at 4°C when exposure times are 60 minutes or less.

Curves C-3 and D-3 in Figure 5 show the curvatures obtained when stimulation in the horizontal position was applied at 4°C. As indicated by these curves, development for 30 minutes at 22°C induces slightly more curvature than development at 4°C. The opposite is true when the klinostat time is extended to 90 minutes. (See Curves C and D in Figure 3.)

Curves C-4 and D-4 in Figure 6 show that 1×10^{-4} M TCBA plus 30 minutes of development on the klinostat produced essentially the same curvature as the identical regimen in the absence of TCBA when geotropic stimulation was applied at 4°C. (Also see Curves C-3 and D-3 in Figure 5.) In the presence of TCBA, 90 minutes of klinostat development time at 4°C produced more curvature (Curve C-1 in Figure 3) than 30 minutes of klinostat time under similar conditions (Curve C-4 in Figure 6.)

TCBA inhibited geotropic bending when stimulation was applied at 22°C. Comparison of Curves A-4 and B-4 in Figure 6 with Curves A-3 and B-3 in Figure 5 verifies this observation. When geotropic stimulation was applied at 4°C and the klinostatic development at 22°C, 1×10^{-4} M TCBA produced the same curvature with either 30 or 90 minutes of development time (Curves D-1 and D-4).

Discussion

The role of 2,3,6-trichlorobenzoic acid as an inhibitor of geotropic curvature of plant stems has been reported in only a few instances. Jones, Metcalfe, and Sexton (3) found that TCBA suppressed the typical geotropic responses in rye grass and Vander Beek (8) observed that certain concentrations of this compound interfered with the upright growth of shoots of cucumber, barley, oats, and other seedlings. In a previous study (Schrank 6) using 15 mm. apical sections of *Avena* coleoptiles, it was discovered that some concentrations of TCBA, notably 1×10^{-4} M, stimulated elongation but inhibited negative geotropic curvature. The combination of facts presented in this paper (Schrank 6) indicated that the primary effect of TCBA was to inhibit the geotropic perception mechanism. There was also some indication that higher concentrations of TCBA under certain conditions had a slight secondary masking effect. In these instances the concentration of the combined growth substances, endogenous auxin plus the TCBA, was so high that the magnitude of the difference in concentrations on opposite sides was thereby reduced. This situation contributed to a small inhibition of curvature.

Results in the previous paper (Schrank 6) indicated that 1×10^{-4} M TCBA did not inhibit nor stimulate elongation of apical coleoptile sections kept in the vertical position at 4°C. However, as shown by Curve II-KT in Figure 1, TCBA caused a small but significant stimulation of elongation at 4°C, especially during the first 12 hours of growth on the klinostat. At 22°C, growth on the klinostat in the presence of TCBA was stimulated by only a very small amount during the first 8–10 hours of growth. The significance of this stimulatory effect of TCBA on coleoptiles growing on the klinostat is not apparent at the present.

When geotropic curvatures of *Avena* coleoptiles are permitted to develop on the klinostat for 90 minutes after stimulation in horizontal position, several facts become apparent. If the geotropic stimulation is applied at 4°C, 1×10^{-4} M TCBA induced no inhibition of geotropic curvature regardless of whether the development was at 4° or at 22°C. For the shorter geotropic exposures (up to 60 minutes when the development was at 4°C and up to 100 minutes when the development was at 22°C) the TCBA caused increased curvatures. This effect, most likely, was due to the growth promotion influence of the TCBA.

Application of geotropic stimulation at 22°C, in the presence of TCBA, resulted in somewhat different responses. With klinostatic development at 22°C, TCBA caused an increase in bending for exposures up to 90 minutes. This again must be due to the stimulation of elongation by TCBA. For longer

geotropic exposures, TCBA was inhibitory to curvature (see Figure 4). Such inhibition is a reflection of the effect of TCBA on geotropic perception. With klinostatic development at 4°C, 1×10^{-4} M TCBA caused an extensive inhibition of upward curvature. This substantiates the observation that the geotropic perception mechanism is more sensitive to TCBA at higher temperatures. One $\times 10^{-5}$ M TCBA plus klinostatic development at 4°C resulted in stimulation of curvature for exposures up to 90 minutes and inhibition of bending for longer stimulation times. An explanation for this sequence has already been given.

Reduction of the klinostat development time from 90 to 30 minutes produced larger curvatures when stimulation was applied at 22°C (Figure 5). TCBA with geotropic stimulation at 4°C had no effect on curvature under these conditions. When the geotropic stimulation was applied at 22°C, 1×10^{-4} M TCBA caused a general inhibition of upward bending. The only instance of stimulation was noted when the horizontal exposure was for 10 minutes at 22°C and the development was carried out for 30 minutes at 4°C. This apparently is a reflection of the fact that TCBA is stimulatory to elongation at 4°C on the klinostat.

The results presented in this paper are consistent with the thesis that the increased geotropic curvatures of 15 mm. apical *Avena* coleoptile segments produced by TCBA are mediated by the stimulatory effect of this compound on elongation. The observed depression of upward bending by 1×10^{-4} M TCBA supports the previous conclusion that this compound also has an inhibitory effect on the mechanism of geotropic perception.

Summary

1. *Avena* coleoptile segments, in the vertical position and on a horizontal klinostat rotating at 1 rpm, elongated at the same rate for 24 hours at 4°C and at 22°C. Two,3,6-trichlorobenzoic acid (TCBA) (1×10^{-4} M) increased elongation of coleoptiles for the first 12 hours at 4°C on the klinostat but caused only a slight increase in growth during the first 8 hours at 22°C.

2. Geotropic curvature of coleoptile segments continuously maintained in the horizontal position increased with increases in temperature from 4°C to 22°C. The Q_{10} for 4°—22°C after 2 hours of curvatures was 2.9. After 4.5 hours of curvature the Q_{10} was 2.0.

3. When coleoptiles were geotropically stimulated at 22°C and subsequently developed for 90 minutes on the klinostat, development at 4°C produced larger curvatures for all exposure times than similar development

at 22°C. The temperature during klinostatic development has no effect on geotropic bending of coleoptiles that were stimulated at 4°C.

4. With geotropic stimulation at 4°C and development at 22°C, 1×10^{-4} M TCBA increased geotropic curvatures for horizontal exposures up to 100 minutes. At a development temperature of 4°C, TCBA increased bending for exposures from 20 to 60 minutes.

5. When geotropic stimulation was applied at 22°C, 1×10^{-4} M TCBA increased curvature for horizontal exposures up to 90 minutes if klinostat development was at 22°C. With development at 4°C, 1×10^{-4} M TCBA caused an extensive inhibition of bending for geotropic exposures up to 180 minutes.

6. Reduction of the klinostat development time to 30 minutes, with horizontal exposure at 22°C, produced maximum curvatures for exposure times up to 90 minutes. Klinostat development at 22°C induced larger curvatures for exposures from 10 to 60 minutes, while development at 4°C was more effective for horizontal exposures from 60 to 90 minutes. When the geotropic stimulation was applied at 4°C, klinostat development at 22°C resulted in slightly larger curvatures for all exposure times than similar development at 4°C.

7. TCBA (1×10^{-4} M) had no effect on geotropic curvature when the horizontal exposures were made at 4°C and the klinostat development time was limited to 30 minutes. Geotropic curvatures were inhibited by TCBA when stimulation was applied at 22°C. Klinostat development for 30 minutes at 22°C gave slightly more curvature than similar development at 4°C.

8. The results herein reported are consistent with the previous conclusion that TCBA apparently inhibits geotropic bending of apical *Avena* coleoptile segments primarily by affecting the gravity perceiving mechanism. Stimulation of bending depends on the increased elongation mediated by TCBA.

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The Uptake of Halide Ions and Their Relationships in Absorption

By

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Over the past decade the ion uptake of different plant cells has generally been discussed by making use of the carrier theories. Based on Epstein's suggestions (1956), the plants have been believed to have a separate uptake system for each group of similar ions. The ions that are found in the same column of the periodic system can sometimes be divided into two uptake groups and ions absorbed by means of similar carriers reciprocally inhibit each other's uptake. The view in question has been supported by experiments, a large proportion of which were made with alkali and alkali earth metal cations, in which the relationships of ions were studied only in one direction and at a relatively limited range of concentration for methodological reasons. That is why Sutcliffe (1957) has been able to point out that the reciprocal relationships of alkali ions can be of different character and according to Fried and Noggle (1958) two systems can play a role in each alkali cation uptake. Apart from the occasional investigation of the uptake relationships of certain ion-pairs (*e.g.* sulphate-selenate, phosphate-arsenate), systematic experiments — similar to those made with alkali cation absorption — have not been made so far with the classes of the periodic system containing anions. Of these the halogen group seems to be most suitable for experiments of this type.

The experiments reported in this paper have been made in the years 1958/59 and 1960, and a part of the initial results obtained was published earlier (Böszörményi and Cseh 1958, Böszörményi, Cseh and Gáspár 1959).

Materials and Methods

Seedlings of winter wheat, variety F. 481, were used in our experiments. The seeds were sterilized with hydrogen peroxide and then germinated in a thermostat for two days in the dark at a temperature of 26°C. The seedlings were transferred to perforated polyvinyl discs and then placed upon the surface of a tenfold-diluted Hoagland solution. Thereafter the plants were grown for two more days in the dark in nutrient solution at 21°C. The 4-day-old plants were used together with the perforated discs (A and B series).

In the experiments made with excised roots, the seeds were germinated in the same way; the only difference being that in this case the seedlings were grown for an additional day in the thermostat and after having rinsed the excised roots with distilled water for a short time, we used them in B series.

A-series. — The experiments with intact seedlings were made in Lundegårdh vessels. As usual, the vessels were continually aerated during the 24 hour uptake period and kept in saturated atmosphere at 21°C. The solutions had been prepared so that they would make possible the determination of the uptake of 4 halide ions in 5—5 concentrations (0, 0.5, 5, 25 and 50 m.equiv./l.). For making up the solutions, the respective K salts of the halide ions had been used. With a view of investigating the relationships, the 4 halide ions were combined by twos in all the possible variants. Considering the 5 concentrations adopted, we had a total of 150 variants. We used 7 seedlings in 100 ml. of each solution.

At the termination of the 24 hour experiment, the roots were washed with distilled water and cut off. Then the shoots and roots were dried separately at 150°C and analyzed. The seeds were separated and their composition has not been investigated. We burned the pulverized, dried samples in oxygen gas — in accordance with the Schöniger's method (1955). The precipitation titration was made with AgNO₃ by means of Methrom E 166 titrscope. It should be noted that some samples having been acidified developed iodine, which was dissolved in chloroform and photometrated, or all the samples susceptible of developing iodine were reduced in basic solution by Dewarda alloy.

The fluoride contents was photometrically determined — following the method adopted by Monnier *et al.* (1948) — in one aliquot of the solution obtained after burning. Because of the low uptake of the fluoride ion, this method turned out to be inadequate at lower fluoride concentrations, since no measureable absorption could be detected.

B-series. — In this series we investigated the effects of halide ions on ¹³¹I-iodide and ⁸²Br-bromide uptake in one hour absorption period.

The excised roots of 20—30 3 day old seedlings were placed into the solution of each of the variants having a concentration 1—25 m.equiv./l. I⁻, the activity of which was 20 μC in 100 ml. Besides the iodide, chloride or bromide with a concentration of 10 m.equiv./l. was also added in parallel variants. During the uptake period the vessels were sunk in 20°C waterbath and were aerated. At the end we washed the roots twice with 50 ml. distilled water and then we transferred them to 3 ml. 96 % ethanol. The fixed roots were then ground with quartz sand, rinsed with 2 ml. 80 % ethanol, transferred to a filter plate and sucked off. The material was washed twice with 2 ml. 80 % ethanol and twice with 2 ml. distilled water. Then the filtrates were united and aliquots were pipetted into sample pans.

The procedure was similar with ⁸²Br-bromid. The activity used generally was

between 10 and 20 $\mu\text{C}/100\text{ ml.}$ in the different experiments. In this case the ground material was filled up to ten ml. and aliquots were taken directly from this suspension for making samples. We used this simpler method, because in the preliminary experiments we could not find the slightest traces of organically bound bromine and therefore we did not run chromatogramms from the extracts, as in the ^{131}I experiments (Böszörményi, Cseh and Gáspár 1959).

The activity of the samples was measured by means of a 1.3 mg/cm.^2 end-window GM tube.

Results

A-series. — At the outset the accumulation of 4 halide ions from solutions of 50 m.equiv./l. concentration in the 24 hour experiments seems to be worth comparing. The high bromide accumulation, which surpasses that of the chloride uptake, is quite surprising (Table 1). It is noteworthy that counting the original chloride contents, the 24 hour bromide and chloride accumulation is essentially the same, whereas that of the iodide is lower, and the fluoride uptake is very low. An interesting result can be obtained by expressing the amount accumulated in the roots as the percentage of the total uptake. It reflects that the relative accumulation in the roots gradually increases from the fluoride to the iodide, that is to say the translocation decreases.

In an experiment performed before the main series, the time course of absorption had also been determined by means of chloride, bromide and iodide solutions of 25 m.equiv./l. concentration (Table 2). The conclusion can be drawn that under the conditions employed in the A-series, the accumulation taking place in the roots quickly becomes saturated (within a period of 6 to 12 hours), while the translocation into the shoots does not come to an end, and the total uptake shows a linear curve up to the end of the experiment. The conditions, particularly the changing of humidity, led to some irregularities in the curve.

Table 1. *The uptake of halide ions by intact wheat seedlings from 50 m.equiv./l. solutions in 24 hour experiments. ($\mu\text{equiv.}$ of halide ions taken up by 7 seedlings.)*

Ion	Roots	Shoots	Whole plants	Accumulation in the roots as the % of the total
F^-	0.84	5.90	6.74	12
Cl^-	6.83/8.88/	38.83/46.55/	45.66/55.41/	15
Br^-	9.93	45.13	55.06	18
I^-	5.25	19.97	25.22	20

(In brackets the total chloride content.)

Table 2. *The uptake of halide ions by intact wheat seedlings from 25 m.equiv./l. solutions in 6—30 hour experiments. (μ equiv. of halide ions taken up by 7 seedlings.)*

Ion	Hours	Roots	Shoots	Whole plants	Accumulation in the roots as the % of the total
Cl ⁻	6	1.5/3.7/	4.35/11.22/	5.85/14.92/	25
	12	4.0	11.33	15.33	26
	18	3.9	17.23	21.13	18
	24	3.5	19.83	23.33	15
	30	4.0	39.20	43.20	9
Br ⁻	6	4.59	7.82	12.41	36
	12	4.09	15.75	19.54	20
	18	6.93	25.65	32.58	21
	24	5.26	24.80	30.06	17
	30	5.59	31.20	36.79	15
I ⁻	6	2.26	4.90	7.16	31
	12	2.18	6.66	8.84	24
	18	3.96	13.49	17.45	22
	24	3.35	14.86	18.19	18
	30	2.86	17.84	20.70	13

(In brackets the total chloride content.)

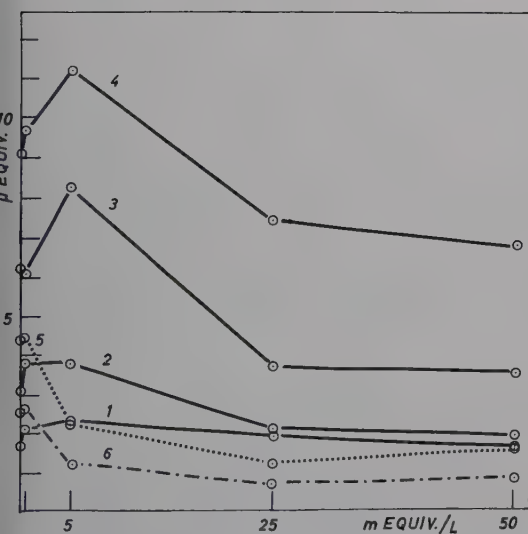


Fig. 1 A.

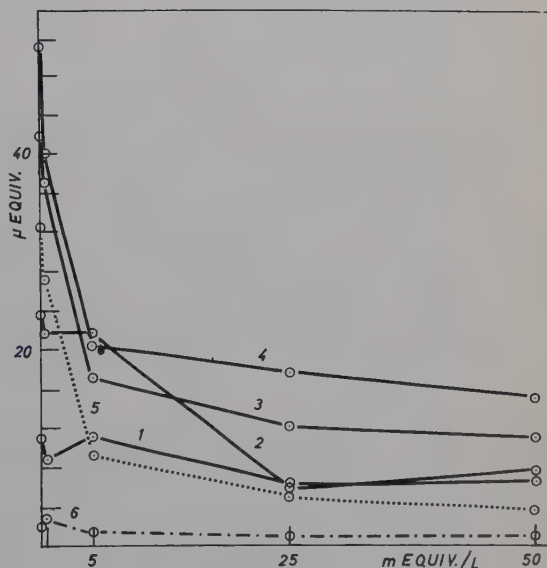


Fig. 1 B.

Figure 1. *The effect of fluoride ion on the uptake of halide ions by intact wheat seedlings from 0—50 m.equiv./l. solutions in 24 hour experiments: A accumulation in the roots, B accumulation in the shoots. 1=0.5, 2=5, 3=25, 4=50 m.equiv./l. Cl, 5=25 m.equiv./l. Br, 6=25 m.equiv./l. J.*

Table 3. *The content of Cl and Br in the roots in the Cl-Br series.*
 μ equiv. per 7 seedlings.

A. Cl-content						B. Br-content				
Cl m. equiv./l.	Br m.equiv./l.					Br m.equiv./l.				
	0.0	0.5	5.0	25.0	50.0	0.0	0.5	5.0	25.0	50.0
0.0	1.50	0.73	1.40	1.04	1.09	0	0.62	3.47	7.00	10.74
0.5	3.09	2.51	2.50	1.21	1.28	0	0.65	2.96	5.27	8.00
5.0	3.01	2.62	2.32	1.39	1.20	0	0.28	2.36	7.24	9.72
25.0	6.13	4.68	4.35	2.23	2.27	0	0.30	2.31	6.53	9.20
50.0	8.64	8.03	6.76	4.58	4.03	0	0.38	3.23	7.29	10.78

And now for the relationships of ions. Low fluoride concentrations (0.5—5 m.equiv./l.) increase the chloride accumulation taking place in the roots of intact plants, while the reverse could be detected in the shoots at every concentration (Figure 1). This particular effect of the fluoride ion is limited to the chloride only, because the bromide and iodide accumulation is inhibited by the fluoride in the whole range of concentrations in both the roots and shoots. It is to be regretted that because of the low rate of fluoride uptake, the investigation of the reciprocal effects cannot be regarded as completed. Based on the data obtained so far we think it probable that all the three halides are capable of reducing the fluoride accumulation of seedlings.

There has been no considerable effect produced on the bromide contents of the roots by the chloride; the results, however, are not uniform. At lower chloride levels minor inhibition could be detected (Tables 3 and 4). At the same time the inhibitive effect of the chloride on the bromide contents of the shoots and of the whole plants is outspoken. The reciprocal effect proved uniformly inhibitive in both the root and shoot fractions.

The stimulating effect of chloride on the iodide contents of the roots is quite noticeable and it can be detected in the whole concentration range

Table 4. *The content of Cl and Br in the shoots in the Cl-Br series.*
 μ equiv. per 7 seedlings.

A. Cl-content						B. Br-content				
Cl m. equiv./l.	Br m.equiv./l.					Br m.equiv./l.				
	0.0	0.5	5.0	25.0	50.0	0.0	0.5	5.0	25.0	50.0
0.0	7.93	7.26	5.55	8.07	7.60	0	2.64	21.08	32.72	47.01
0.5	12.18	12.09	9.80	6.99	7.33	0	1.77	17.49	32.36	32.54
5.0	17.63	15.51	13.78	11.29	10.19	0	1.14	10.86	30.92	45.87
25.0	32.38	31.41	28.25	19.00	14.18	0	1.44	9.26	23.36	32.30
50.0	46.93	47.44	36.36	24.08	20.58	0	0.97	7.88	19.64	29.67

Table 5. *The content of Cl and I in the roots in the Cl-I series.*
 μ equiv. per 7 seedlings.

A. Cl-content

Cl m. equiv./l.	I m.equiv./l.				
	0.0	0.5	5.0	25.0	50.0
0.0	2.31	1.71	2.41	1.88	2.68
0.5	4.34	3.02	2.05	1.80	1.39
5.0	4.67	3.57	3.24	2.03	2.14
25.0	7.18	5.38	6.45	4.07	3.39
50.0	8.89	8.91	6.50	6.77	5.28

B. I-content

I m.equiv./l.				
0.0	0.5	5.0	25.0	50.0
0	0.37	1.74	6.10	6.01
0	0.40	1.94	6.30	5.98
0	0.64	2.53	5.37	7.09
0	0.89	3.62	5.52	7.64
0	1.10	5.49	5.81	9.49

Table 6. *The content of Cl and I in the shoots in the Cl-I series.*
 μ equiv. per 7 seedlings.

A. Cl-content

Cl m. equiv./l.	I m.equiv./l.				
	0.0	0.5	5.0	25.0	50.0
0.0	8.67	6.04	5.30	6.30	5.02
0.5	8.26	8.30	7.15	7.11	6.87
5.0	19.86	16.00	11.91	7.97	7.06
25.0	32.40	28.35	21.11	18.40	16.82
50.0	41.87	44.22	29.74	21.27	21.87

B. I-content

I m.equiv./l.				
0.0	0.5	5.0	25.0	50.0
0	0.51	4.26	17.34	19.33
0	0.61	4.62	19.54	21.04
0	1.11	7.30	16.77	20.18
0	0.72	6.89	9.81	16.13
0	0.77	7.33	12.21	14.25

Table 7. *The content of Br and I in the roots in the Br-I series.*
 μ equiv. per 7 seedlings.

A. Br-content

Cl m. equiv./l.	I m.equiv./l.				
	0.0	0.5	5.0	25.0	50.0
0.0	0	0	0	0	0
0.5	1.95	0.63	0	0	0
5.0	3.86	2.79	1.14	0.65	0.42
25.0	6.89	6.09	4.24	2.57	2.09
50.0	11.92	8.14	6.95	5.11	4.67

B. I-content

I m.equiv./l.				
0.0	0.5	5.0	25.0	50.0
0	0.40	2.01	3.27	6.41
0	0.57	1.68	4.16	5.30
0	0.46	2.62	5.16	5.58
0	0.29	2.11	4.33	5.31
0	0.32	1.83	3.62	7.64

Table 8. *The content of Br and I in the shoots in the Br-I series.*
 μ equiv. per 7 seedlings.

A. Br-content

Cl m. equiv./l.	I m.equiv./l.				
	0.0	0.5	5.0	25.0	50.0
0.0	0	0	0	0	0
0.5	2.97	0	0	0	0
5.0	17.60	10.52	5.00	3.25	1.20
25.0	30.10	29.52	19.50	12.58	8.32
50.0	47.05	37.50	38.92	22.05	13.90

B. I-content

I m.equiv./l.				
0.0	0.5	5.0	25.0	50.0
0	0.59	3.80	13.98	23.06
0	1.08	3.52	19.68	23.30
0	1.19	6.87	18.47	23.97
0	1.41	6.71	16.35	19.34
0	0.90	6.64	15.14	16.06

Table 9. *The uptake of ^{131}I -iodide by excised wheat roots from carrierless solution and from a solution with 2.5 m.equiv./l. carrier in 3 hour experiments. (The activity of successive extract portions in counts per minute; from the roots of 20 seedlings.)*

Carrier	80 % ethanol					Distilled water			
	1	2	3	4	5	1	2	3	4
without...	28170	3056	319	112	145	267	174	172	159
with	33240	3892	740	178	44	102	55	62	57

Carrier	0.2 N NaOH					Total extractable	The rest
	1	2	3	4	5		
without...	13857	6096	1588	332	154	54601	724
with	591	348	95	31	76	39511	99

investigated. As to the shoots, the chloride influence does not spread over the whole range of concentration, since by raising the chloride concentration, the iodide contents increases for a time, then decreases. The iodide reduces chloride accumulation in both the roots and shoots.

The iodide accumulation can generally be intensified by adding lower bromide concentrations, while higher bromide levels produce the reverse effect. It should be noted that we failed to detect this connexion when using the highest iodide level. The above described relationship can be observed in the shoot fractions too, where it was fairly detectable at every concentration used. And finally the iodide inhibited the bromide accumulation both in the shoots and roots to the same extent as has been seen with the chloride.

B-series. — As is known from the literature on this subject, there is a considerable difference between the behaviour of carrier-free isotope solutions and those with carrier. Considering the fact that under natural field conditions the absorption of tracer elements and iodide takes place from solutions of very low concentration, we started making experiments in this direction by using ^{131}I -iodide. First the extractability of absorbed ^{131}I was investigated in carrier-free solutions and in solutions of 2.5 m.equiv./l. concentration in 3 hour experiments. As has been mentioned in the chapter Materials and Methods, the roots washed with distilled water were ground in 3 ml. 96 % ethanol, and then 4—4.2 ml. 80 % ethanol, distilled water and 0.2 N NaOH washing solution fractions were collected. The activity of each fraction was determined separately (Table 9). The experiences obtained can be summarized as follows: a large proportion of the quantity absorbed can be extracted by means of 80 % ethanol, further washing with distilled water slightly increases the quantity extracted, and finally, after the basic extrac-

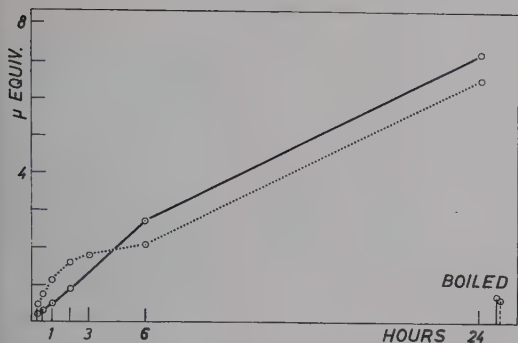


Fig. 2 A.

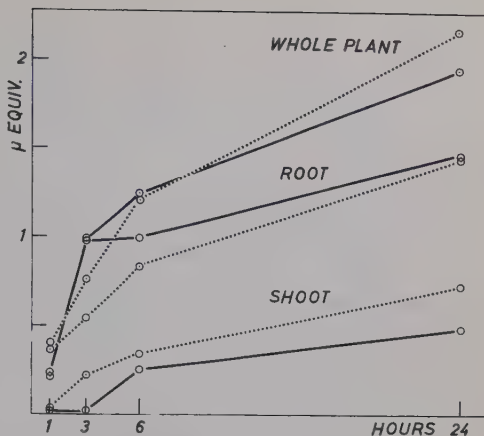


Fig. 2 B.

Figure 2. The uptake of ^{131}I -iodide by excised wheat roots (A) in 15'–24h experiments and by intact seedlings, (B) in 1–24 hour experiments. Diacritic symbols: \odot — from carrier-free ^{131}I -iodide solution in μC , $\odot\cdots$ from 2.5 m.equiv./l. solution in μequiv .

tion, the activity of the residue is negligible. Considerably greater activity can be obtained from water, and particularly from basic fractions than from those of the alcohol when using carrier-free solutions.

In view of the results, the question can be raised whether the iodide taken up from the carrier-free solutions does have different characteristics from the point of view of the plant from those absorbed from iodide solutions of higher concentrations. Absorption time courses of excised roots or of intact seedlings may serve as certain points of support in answering the problem. With the excised roots we used either carrier-free solutions of 100 $\mu\text{C}/100\text{ ml}$. or solutions of 20 $\mu\text{C}/100\text{ ml}$. 2.5 m.equiv./l. Seven variants were investigated in both series for a period of 15 minutes to 24 hours. For the purpose of investigations, separate control variants consisting of roots killed by boiling for 24 hours were used. With the intact seedlings 100 μC activity in a solution of 100 ml. was employed in both experiments and the 2.5 m.equiv./l. concentration acted as carrier in the second one. The uptake period ranged between 1–24 hours and the roots and shoots were analyzed separately.

Taking into consideration absolute values, there has naturally been a great difference with regard to the quantities absorbed; moreover a considerable part of the activity can only be extracted by means of basic solution when using carrier-free solution; nevertheless the curve of the time course, or the distribution of the activity between the roots and shoots was essentially the same in both methods (Figure 2). It is believed that from these data the

Table 10. *The uptake of ^{131}I -iodide and ^{82}Br -bromide by excised wheat roots from 1—25 m.equiv./l. solution in one hour experiments. (^{131}I uptake in $\mu\text{equiv.}$; ^{82}Br uptake in cpm in an 1/100 aliquot.)*

Concentration m.equiv./l.	$^{131}\text{I}^-$ uptake			$^{82}\text{Br}^-$ uptake		
	control	10 m.equiv./l.		control	10 m.equiv./l.	
		Cl^-	Br^-		Cl^-	I^-
1.0	0.41	1.66	0.64	287	196	175
2.5	1.09	1.50	1.27	214	190	195
5.0	3.31	2.15	3.43	177	201	165
10.0	3.50	3.25	3.22	212	214	201
25.0	7.19	7.67	7.52	200	143	145

conclusion can be drawn that the decrease of uptake in time is not so much of saturation character, but it can be linked up with some of the physiologic changes of the experimental material.

It is worth noting that the iodide "absorption" of boiled roots used as control showed a very low level in both solutions, since after 24 hours it was far below the uptake of intact roots within a period of half an hour.

As for the relationships of halide ions, this series confirmed data obtained in the A-series. The chloride and bromide stimulated the iodide uptake of excised roots even in the one hour absorption period. This influence, however, could only be detected in low iodide concentrations (Table 6). The chloride and iodide were inhibitive on the uptake of ^{82}Br -bromide in the whole range of concentrations.

Discussion

The similar behaviour of the chloride and bromide and their competitive effect on each other's uptake have been known since Hoagland *et al.* (1928) pointed out that the presence of the chloride decreases the accumulation of Nitella in bromide, while the sulphate, nitrate and phosphate ions do not have a considerable influence. The results of the experiments made by Epstein (1953) are in accordance with those of Hoagland, since he demonstrated the inhibitive effect of the chloride on the bromide uptake of excised barley roots and confirmed its competitive character; while the effect of the nitrate was found to be weak and of a different nature. The differences in the chloride and nitrate uptake were underlined by Stenlid (1957) too. Based on his experiments, Epstein put forward the idea that the chloride and bromide absorption takes place in a carrier system which differs from the nitrate uptake system. Recent results, however, obtained by Lundegårdh (1959),

according to which the phosphate and sulphate increase the chloride uptake in potato slices, the nitrate has the same effect but only at the "initial" stage because further chloride uptake is inhibited by the nitrate, contradicts Epstein's conclusions to a certain extent. At the same time the chloride inhibited the nitrate absorption in wheat roots.

The relationships between the chloride-iodide and bromide-iodide were first investigated by Hoagland *et al.* (1928) and they found the iodide to have reduced the accumulation of bromide by *Nitella*. In contrast to what had been demonstrated by Hoagland, the iodide was reported to have stimulated chloride accumulation (Lewis and Powers 1941); while the iodide contents were said not to be affected by the chloride concentration of the solution.

Sparse data are available with regards to fluoride uptake, and none of them refers to the relationships between other halide ions.

Data obtained in our experiments are unanimous in confirming the reciprocal chloride-bromide relationships of inhibitive character. At the same time we have found — in contrast to what was suggested by Lewis and Powers (1941) — that the chloride and to the same extent the bromide stimulate the iodide absorption of excised roots as well as intact seedlings. The inhibitive reciprocal effect, detected in our experiments, corresponds to the inhibitive influence referred to by Hoagland.

As far as we know, we have been the first to investigate the stimulatory effect of the fluoride ion on the chloride accumulation, which takes place in the roots of intact seedlings. It is of interest that the iodide and bromide are not in the least stimulated.

In view of what has been found, we have come to the conclusion that the relationships of halide ions are more complicated than expected. No doubt the carrier theory can be some kind of an explanation for the stimulatory effect of ions on each other's uptake (*e.g.* Kahn and Hanson's interpretation of the Viets effect), yet such a speculation based only on kinetic considerations does not seem applicable in our view for the full interpretation of the varied relationships detected. It is very probable that a part of the influences demonstrated does not take effect directly through the carrier systems (*e.g.* the inhibitive influence of the iodide and the different stimulatory effects).

Summary

The uptake of halide ions and the relationships of absorption have been investigated by using wheat seedlings grown in the dark and the excised roots of seedlings. From the point of view of the rate of uptake, the halide ions can be ranked as follows: $F < I < Cl < Br$. The fluoride ion stimulates

the chloride accumulation, which takes place in the roots of the seedlings, but it reduces the total absorption of the seedlings. We have not been able to detect the stimulatory influence of the fluoride on the bromide and iodide absorption. The chloride and bromide ions have been found to have inhibited each other's uptake. On the other hand, both ions appeared to have stimulated iodide absorption in different experiments made with isolated roots and seedlings, and the iodide inhibited the uptake of both of them. The extractability of carrier-free ^{131}I -iodide and when used with carrier is different, but the two forms resulted in similar absorption time courses and there were no differences to be found in the translocation of ^{131}I taken up from the two solutions in experiments with intact seedlings. Very little ^{131}I absorbed from carrier-free solutions was bound in boiled roots.

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Interactions between Chromophore and Protein in Phycoerythrin from the Red Alga *Ceramium rubrum*

By

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Introduction

Svedberg and Katsurai (1929) designated the phycoerythrins isolated from red algae as R-phycoerythrin since their absorption maxima at 495, 540, and 565 m μ were different to the phycoerythrins isolated from members of the blue-green algae. In recent years, however, other phycoerythrins have been isolated from red algae and their absorption characteristics have been noted to vary greatly from the typical R-phycoerythrin (Haxo, O'hEocha and Norris 1955, Airth and Blinks 1956, O'hEocha 1958).

Lemberg (1930) proposed that the tetra-pyrrole chromophore, namely phycoerythrobilin, was identical to mesobilirhodin, and was strongly attached to the protein; the linkage probably involving pyrromethane groupings in addition to primary peptide bonds. This, however, has not been demonstrated. On examining several phycoerythrins of different absorption spectra, O'hEocha (1958) found that they all contained one and the same chromophore, and no evidence was obtained to indicate a relationship between phycoerythrobilin and mesobilirhodin. He therefore considered that differences in the globulin structure of the protein or in the protein-chromophore linkages may account for the great spectral variation. Studies of the amino acid composition of phycoerythrins from several red algae by Fujiwara (1956), Jones and Blinks (1957), and Kimmel and Smith (1958) do indicate some differences in the composition of the chromoproteins. The work of Fujiwara, and Kimmel and Smith also indicated that the sulfur content of the phycoerythrin was greater than that accounted for by the sulfur amino

acids. Fujiwara (1957) determined the amino acid composition of the chromopeptides derived from the phycoerythrin of *Porphyra tenera* by peptic digestion. She obtained 16 peptide fractions and found that cystine was common to each one. The author therefore suggested that the excess sulfur in phycoerythrin may be concerned in the linkage between the chromophore and protein via thioether bridges as reported by cytochrome C by Theorell (1938) and Tuppy and Paleus (1955).

The present paper is concerned with evidence to support the concept that interactions between chromophore and protein play an important role in contributing to the absorption spectrum of phycoerythrin in the red alga *Ceramium rubrum*.

Materials and Methods

The red alga *Ceramium rubrum* was freshly collected, washed twice with distilled water and finally covered with distilled water and frozen at -20°C for 48 hours. The alga was then brought to room temperature and the phycobilin pigments were allowed to extract over a period of 2 days at 5°C . The material was then filtered through cheese cloth and the filtrate passed through Watman No. 1 filter paper pulp. The highly fluorescent filtrate was centrifuged at $5000 \times g$ for 30 minutes, and the chromoproteins were precipitated from the supernatant with ammonium sulfate at 50 % saturation. The purple-red precipitate was redissolved in distilled water and dialysed against running tap water for 24 hours followed by 0.1 *M* acetate buffer pH 5.0 for a further 24 hours at 5°C . The nondialysable pigment solution was concentrated by pervaporation. The individual pigments were resolved by chromatography on tri-calcium phosphate and celite columns and the phycoerythrin eluted with 1 *M* acetate buffer pH 5.0 (Jones and Blinks 1957). The isolated phycoerythrin was brought to a pH of 7.0 before study by dialysing against 0.1 *M* phosphate buffer pH 7.0. All reactions were studied at this pH unless otherwise stated.

The phycoerythrin was subjected to the following treatments: heat denaturation, pepsin hydrolysis, 6 *M* urea, 4 *M* guanidine HCl, saturated *p*-chloromercuribenzoate, 0.01 *M* sodium hydrosulfite, 1 *M* hydrazine hydrate, 1 % hydrogen peroxide, and U.V. irradiation. The absorption spectra were recorded at appropriate time intervals thereafter. Control samples of phycoerythrin were recorded with each experiment.

All experiments were conducted in Pyrex test tubes except those involving U.V. irradiation in which case silica test tubes were used. Absorption data were recorded with a Beckman spectrophotometer using silica cuvettes. The source of ultra violet radiation was a low pressure mercury lamp with a suitable filter to produce radiation with a maximum at 2537 Å.

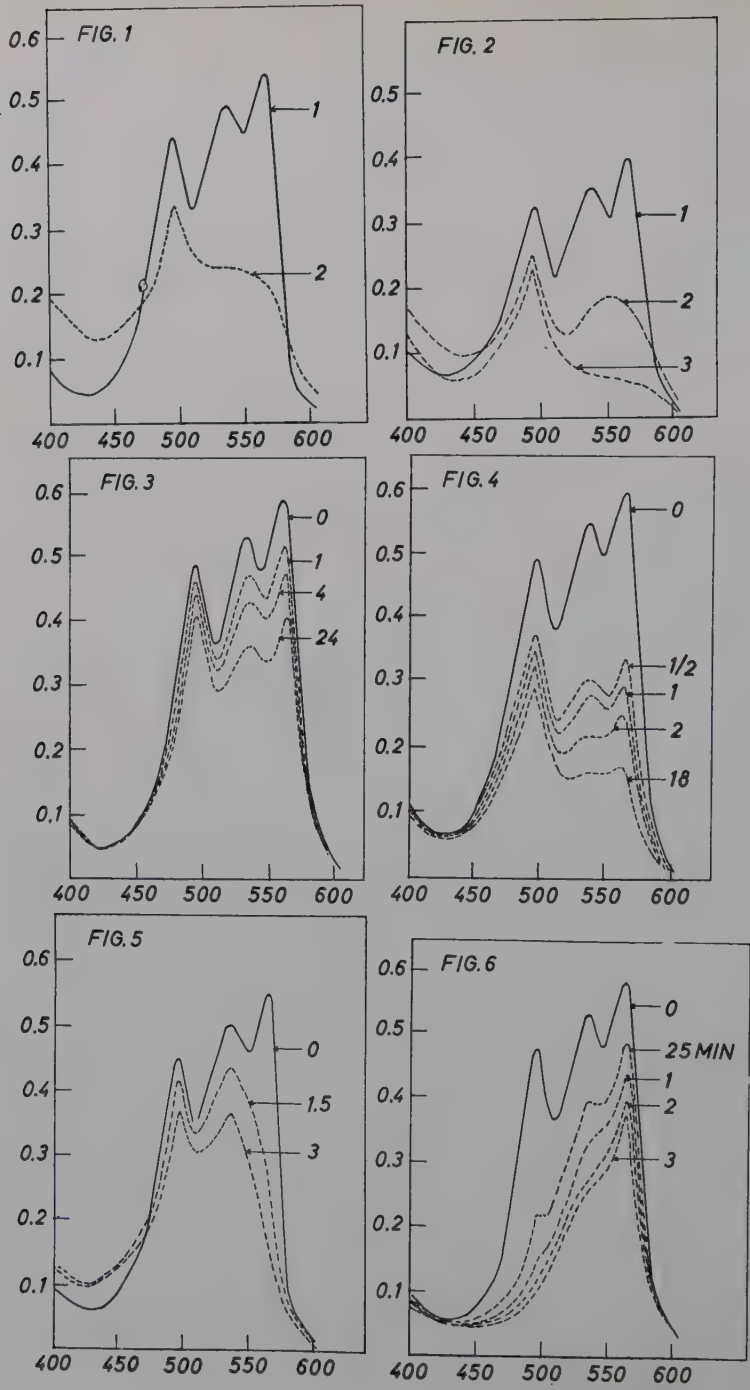
Results and Discussion

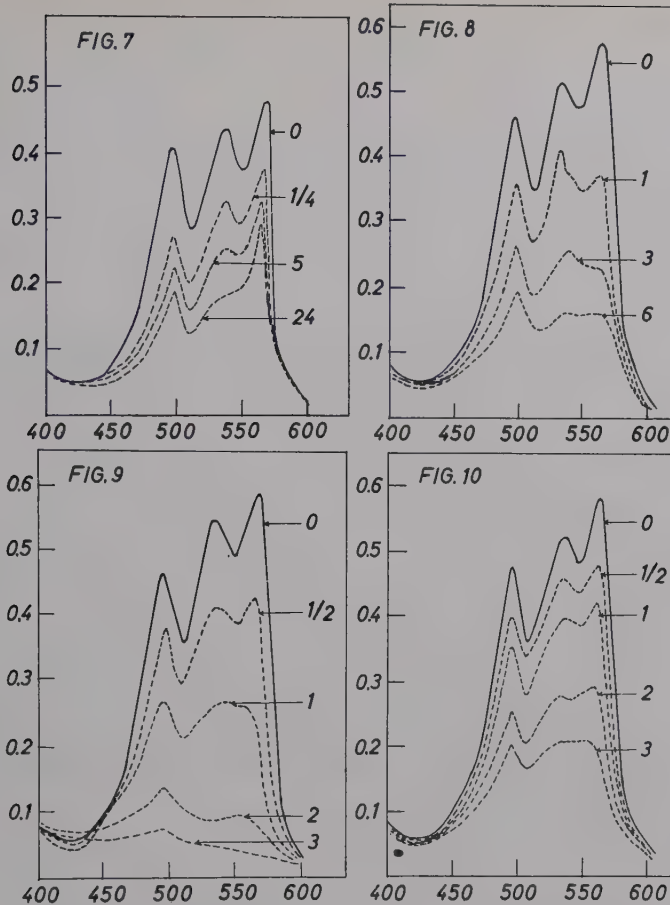
The fact that the characteristic absorption peaks at 540 and 565 mμ are rapidly diminished following a sudden increase in temperature (Figure 1) or treatment with 4 *M* guanidine HCl (Figure 4) indicates that an internal

configurational change in the protein affects this region of the absorption spectrum of phycoerythrin. The effect of guanidine HCl suggests that hydrogen bonds may be involved, although treatment with 6 *M* urea (Figure 3), has only a mild effect. Digestion of the protein moiety of the phycobilin with 0.5 % pepsin at pH 1.6 during an incubation period of 17 hours at a temperature of 37°C, results in almost complete reduction in the absorption at 540 and 565 m μ (Figure 2). It should be noted that under the above mentioned treatments the absorption max. at 495 m μ , which is assumed to be due to the tetra-pyrrole structure of the pigment (Pruckner and Stern 1937), is little affected.

The disappearance of the long wavelength absorption at 565 m μ following treatment with *p*-chloromercuribenzoate (Figure 5) is indicative that sulfhydryl groups of the protein are contributing to this long wavelength absorption. Since this lowest excited state is also responsible for the emission of the fluorescence of phycoerythrin, the interaction of sulfhydryl groups of the protein with the chromophore may be important in the efficiency of energy transfer between phycoerythrin and chlorophyll during photosynthesis in red algae. The strong reducing agent, sodium hydrosulfite rapidly bleaches the 495 and 540 m μ peaks (Figure 6), indicating that the chromophore is reduced and that comparatively stable sulfur linkages, either as disulfide or thioether bonds may be disrupted. A similar effect is noted with the hydrazine hydrate (Figure 7). The stability of the 565 m μ peak under these conditions is in accordance with the concept that this absorption is associated with an interaction between sulfhydryl groups and chromophore since these would not be affected by the reducing agent. Attempts to demonstrate the presence of thioether linkages by the silver sulfate technique used by Paul (1950) for cytochrome C failed. In the presence of 1 % hydrogen peroxide the 565 m μ peak is most unstable (Figure 8). It is apparent therefore, that the tetra-pyrrole pigment and the protein are affected by reduction and oxidation. The ability of -S-S- and -SH compounds to undergo reduction and oxidation, and their ability to combine with other molecules or radicals through the sulfur atom, lends support to the hypothesis that such compounds may be contributing to the 540 and 565 m μ absorption peaks in the phycoerythrin from *C. rubrum*.

Further evidence that disulfide bonds, which maintain the integrity of proteins, may be concerned with the binding of the protein and chromophore is seen from the results of the experiments involving U.V. light. Ultra violet light at 2537 Å bleached the 540 m μ peak and the 565 m μ peak, although the latter occurred at a slower rate (Figure 9). This is of interest since Setlow (1955) and Setlow and Doyle (1957) have demonstrated that U.V. light can break disulfide bonds. The observation that glutathione imparts appreci-





Figures 1—10. On the abscissa wave-length $m\mu$, on the ordinate optical density.

Figure 1. Effect of temperature upon the absorption spectrum of phycoerythrin. 1 control, 2 heated 1 min. 100°C .

Figure 2. Effect of pepsin upon the absorption spectrum of phycoerythrin. 1 control, 2 HCl pH 1.6, 3 Pepsin pH 1.6. All 17 hours, 37°C .

Figure 3. Effect of 6 M urea upon the absorption spectrum of phycoerythrin. Figures denote time in hours.

Figure 4. Effect of 4 M guanidine upon the absorption spectrum of phycoerythrin. As Figure 3.

Figure 5. Effect of saturated *p*-chloromercuribenzoate (PCMB) upon the absorption spectrum of phycoerythrin. As Figure 3.

Figure 6. Effect of 0.01 M sodium hydrosulfite upon the absorption spectrum of phycoerythrin. As Figure 3.

Figure 7. Effect of 1 M hydrazine hydrate upon the absorption spectrum of phycoerythrin. As Figure 3.

Figure 8. Effect of 1 % hydrogen peroxide upon the absorption spectrum of phycoerythrin. As Figure 3.

Figure 9. Effect of short wave ultra violet light upon the absorption spectrum of phycoerythrin. As Figure 3.

Figure 10. Effect of short wave ultra violet light plus 0.01 M glutathione upon the absorption spectrum of phycoerythrin. As Figure 3.

able protection when the chromoprotein is subjected to U.V. irradiation (Figure 10), is further evidence that disulfide bonds are involved in this phenomenon. Little, if any, bleaching by light in the visible range was noted.

The effect of SH-compounds upon the absorption spectra of certain pigments has been described by Fujimori (1955). The author succeeded in bringing about the association of a fluorescent pigment (a derivative of fluorescein) with such SH-compounds as egg albumin, glutathione and cysteine, and noted that the yellow color disappeared and that a shift in absorption spectra to longer wave lengths occurred. The role of SH-groups in the formation of the visual pigment rhodopsin is also of interest (Wald and Brown 1951, 1952). These authors demonstrated that the synthesis of rhodopsin requires the presence of free sulfhydryl groups in the protein opsin, and have suggested that the sulfhydryl groups may be directly involved in the binding of the carotenoid pigment to the protein. During the photobleaching of rhodopsin two sulfhydryl groups were liberated for each retinine molecule formed.

It is apparent therefore that the absorption peaks characteristic of R-phycoerythrin do not result from a single interaction between chromophore and protein, but are dependent upon a number of interactions, some of which may well be due to the participation of disulfide bonds and sulfhydryl groups within the molecule. The difference in the fine structure of proteins and such multiple interaction between chromophore and protein would, in all probability, account for the variation in absorption spectra of phycoerythrins from different sources. This would be true even if the chromophores of all the phycoerythrins were similar.

Summary

The absorption spectrum of the R-phycoerythrin from *Ceramium rubrum* is not only due to the chromophore, but to interactions between the chromophore and the protein. Evidence is presented to indicate that disulfide bonds, sulfhydryl groups and hydrogen bonds are involved in these interactions.

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The Effect of 3-Amino-1,2,4-triazole on Certain Plant Tissues Grown *in vitro*

By

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Introduction

The mechanism of the phytotoxic action of 3-amino-1,2,4-triazole (3-AT) is principally due to its effect on the biological synthesis of riboflavin (17, 18). In the fall of 1959, it was announced by the U. S. Department of the FDA that certain cranberry crops would be condemned if they were found to contain any of the 3-AT which apparently had been misused in its agricultural application (5). Furthermore, it had been inferred that 3-AT might be a carcinogen.

It is interesting to note that 3-AT was actually found to delay carcinogenic action of dimethylaminoazobenzene in the liver of rats (9). Because of these developments, the author considers it important to report on earlier experiments which were done to study the effect of 3-aminotriazole on certain plant tissues (19).

Materials and Methods

The virus tumor tissue of Black's original R₂ strain from *Rumex acetosa* (1) and three tissue cultures of Boston Ivy, *Parthenocissus tricuspidatus*, namely crown gall, habituated, and callus tissues were used in these experiments. Dr. R. M. Klein of the New York Botanical Garden kindly supplied the tissues from the Boston Ivy plant.

The medium (pH 5.2) on which the *Rumex* tissue was grown was that employed by Burkholder and Nickel (3). Uniform pieces of tumor tissue of about 25 mg. fresh weight were placed on the media and the tubes were kept at 25°C under diffuse continuous illumination. After three weeks, final weights were tabulated.

White's nutrient solution (21) was used for the other tissues, naphthaleneacetic acid at a final concentration of 10 $\mu\text{g.}$ per liter being included in the media when callus tissue was employed. The initial weight for the callus and habituated tissue was about 25 mg., but for crown gall it was 50—100 mg. The callus tissue was grown in continuous diffuse light and harvested after four weeks. The crown gall and habituated tissue were not grown under continuous light and were harvested after three and four weeks respectively. Data was obtained from at least 10 tubes per treatment but a few measurements were rejected according to statistical procedures given by Sachs (15). The per cent growth increments then were determined on individual pieces as follows:

$$\frac{\text{fresh weight at 3 weeks}-\text{original weight} \times 100}{\text{original weight}}$$

Results

3-Amino-1,2,4-triazole was found to inhibit the growth of all the tumor tissues as indicated in Table 1. The inhibition of growth by 3-AT was greatest with the crown gall and habituated tissues. At a concentration of 5×10^{-4} M 3-AT, the per cent inhibition was 79, 73, 48, and 43 for crown gall, habituated, callus, and Rumex tissue respectively. Inhibition was greater than 90 per cent for the callus tissue at a concentration of 5×10^{-3} M but less than 10 per cent at a concentration of 7.5×10^{-5} M.

Discussion

On initial inspection of the data, it would appear that the responses of the various plant tissues to treatment with 3-AT indicate some metabolic

Table 1. *The effect of 3-AT on the growth of certain plant tissues grown in vitro.*

3-AT M	Per cent Inhibition of growth			
	Rumex	Crown gall	Habituated	Callus
7.5×10^{-5}	—	—	—	8.3 (1)
1.0×10^{-4}	8.1 (3)	6.9 (4)	29.3 (1)	10.4 (4)
2.0×10^{-4}	—	28.1 (3)	—	23.2 (1)
2.5×10^{-4}	24.5 (3)	—	39.4 (1)	—
3.0×10^{-4}	—	59.4 (2)	—	—
4.0×10^{-4}	—	51.4 (3)	—	—
5.0×10^{-4}	43.0 (3)	78.7 (3)	73.1 (1)	47.6 (3)
7.5×10^{-4}	—	—	66.4 (1)	—
1.0×10^{-3}	—	86.8 (2)	—	70.4 (2)
5.0×10^{-3}	—	—	—	91.1 (1)
1.0×10^{-2}	—	—	—	93.2 (1)

The numbers in parentheses represent the number of runs at the particular molar concentration.

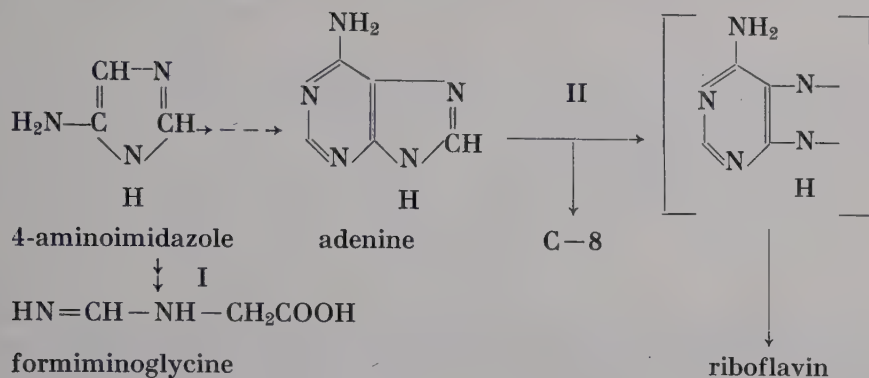
differences, but since two of the less affected tissues were grown under light, these differences may not be real. Rather, the data could indicate that 3-aminotriazole is more toxic under conditions of none or low light intensity or duration. Whether or not riboflavin synthesis is affected in these tissues was not examined. However, in view of the experiments (9) which show that 3-aminotriazole antagonizes the carcinogenic effect of dimethylaminoazobenzene, and in view of recent experiments with streptomycin (14), it is possible that 3-AT is affecting certain enzyme systems involving rupture of N to N and N to C linkages. In view of the statement by Ross (13) that there is not sufficient information on the relative tendencies of heterocyclic ring systems to undergo cleavage, the author hopes that the remainder of this discussion may point to some necessary requirements of anticancer compounds.

Now, while it is evident that 3-AT prevents riboflavin biosynthesis, the exact enzymatic step has, so far, not been reported. In this vein it should also be mentioned that the phytotoxic action of streptomycin is like 3-AT, *i.e.*, it is a growth inhibitor and prevents chloroplast formation (2, 6). On theoretical grounds, if a growth inhibitor effects its activity by blocking the synthesis of a metabolite required at some step in a sequence of biosynthetic reactions, then the effect of such a substance should be circumvented by providing that compound which is a product of the drug-inhibited reaction. In this sense, utilizing tomato plants (17) and *Escherichia coli* (11) the toxic effects of 3-AT and streptomycin, respectively, have been markedly mitigated by simultaneously supplying vitamin B₂ to the system containing either of these growthinhibiting substances.

It would appear that the exact site of action of 3-AT appears to be located in the enzymatic degradation of the 4-aminoimidazole (4-AI) ring occurring as such or as found within the purine ring. In support of this postulation, Rabinowitz and Pricer (12) have shown that 3-AT, indeed, is an antimetabolite for 4-AI since the rupture of the imidazole ring to form formimino-glycine is prevented when 3-AT is included in the enzymatic system obtained from the organism, *Clostridium cylindrosporum*.

Now Roth, Amos, and Davis (14) have demonstrated that when streptomycin is present in a concentration that prevents growth and causes rapid loss of viability, *E. coli* cells excrete into the medium a mixture of 5'-adenylic and 5'-guanylic acids. Since it has been well established that the adenine molecule is virtually incorporated intact into the riboflavin molecule after rupture of the 5-membered imidazole ring of the purine and losing the carbon atom in the 8 position (16); it would seem, by previous mentioned facts, that both streptomycin and 3-AT prevent biosynthesis of vitamin B₂ by inhibiting the enzymatic degradative reaction of adenine. It would appear, then,

that in the following metabolic pathway sites I and II are sensitive to both 3-AT and streptomycin:



A further significance of all of these experiments may point to a real difference in action between plant tissue and normal animal cells and a certain comparison between plant tissue and some animal carcinomas. Since cancer cells are undergoing rapid division, it would seem that purines must be involved. If purines are involved, then their degradation and the utilization of these products is certainly possible. Now while animals primarily obtain their vitamins from plants, it is absolutely necessary for the plants to produce their own vitamins. If a vitamin, like riboflavin, were rapidly being formed by carcinomas within animal tissues, it is conceivable that a wholly independent oxidative system could be set up which rapidly forms excess ATP at the expense of surrounding tissues and simply but literally goes wild within the animal system.

Thus, alternative or additional factors which may account for the effect of 3-AT on certain tissues may be related to the fact that 3-AT has been demonstrated (7) to affect phosphorylase of *Oscillatoria princeps*, and that the glucose adduct of 3-AT has much less affinity for hexokinase than does glucose itself (8). That the inhibition of phosphorylase by 3-AT can be effectively reversed by the addition of manganous or ferric irons (7) may throw some light on the enlargement of the thyroid gland caused by 3-AT since this herbicide has the ability to chelate cations (20). Since thyroxine also has the same ability, it is interesting to consider the speculation that certain of the metabolic effects of the hormone are related to binding of Mg^{++} , Mn^{++} , Zn^{++} , Ca^{++} or Cu^{++} (22). The fact that glucose as the adduct of 3-AT is less affected by hexokinase than glucose itself may be a factor involved in the 3-AT inhibition of growth of plant tissues noted by experiments presented in this paper, since under the culture conditions dextrose may be less available to the tissues in the presence of 3-AT than without it. It is

further interesting to note that the hexosephosphate shunt has been shown to be an active metabolic route in the thyroid tissue (4) and in certain tumors (10), and that the 3-AT adduct seems to affect reactions of C_1 of the hexose chain (8).

The anomalous situation presented here is that 3-AT may be quite important by virtue of preventing growth of certain tissues, yet it has been given the reputation of being carcinogenic.

Summary

1. The herbicide, 3-amino-1,2,4-triazole inhibits the growth of certain plant tissues grown *in vitro*; namely, Rumex, crown gall, habituated, and callus tissues.

2. Although the nature of the mechanism of the growth inhibition of these tissues by 3-AT was not investigated, three possible explanations seem worthy of further investigation; namely,

- a) possible interference of the biosynthesis of riboflavin
- b) possible interference with enzymatic systems containing phosphorylase and hexokinase
- c) chelation effect of certain metals required in enzymatic processes.

3. 3-Amino-1,2,4-triazole should be studied for anticancer activity by virtue of its ability to prevent certain N to N or N to C linkages important to purines and their derivatives.

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The Enzymatic Oxidation of Reduced Diphosphopyridine Nucleotide in the Maize Root Tip by an Oxidation Product of Ascorbic Acid

By

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In recent years several reports have appeared that link H-donating systems on the one hand and ascorbic acid and its oxidase on the other. Conn and Vennesland (1951) and Mapson and Goddard (1951) working independently, almost simultaneously, announced an oxidative pathway which linked the substrate triphosphopyridine (TPNH) through a sequence of enzymatic reactions involving glutathione reductase, dehydroascorbic acid, L-ascorbic acid, and ascorbic acid oxidase. Another nucleotide-ascorbic acid system was demonstrated by Mathews (1951) in which the oxidation of reduced diphosphopyridine nucleotide (DPNH) was brought about by preparations from pea seedlings in cyanide resistant reactions involving ascorbic acid. More recently Beevers (1954), Nason *et al.* (1954), Kern and Racker (1954), and Hackett (1958) have demonstrated the presence of an enzyme in plant materials which catalyzes the oxidation of DPNH by oxygen in the presence of ascorbic acid and its oxidase. Dehydroascorbic acid (DHA) could not replace ascorbic acid, consequently it has been postulated that a labile one-electron oxidation product of ascorbic acid (monodehydroascorbate?) acts as the electron acceptor from DPNH. Kern and Racker (1954) have isolated the enzyme from yeast, and have shown it to be a flavoprotein. The presence of a system linking reduced nucleotides with ascorbic acid and from there to molecular oxygen appears to be well established.

In a previous study concerning the distribution and intracellular localization of ascorbic acid oxidase in the maize root tip it was found that as cells of the meristematic zone expand and elongate there is a concomitant increase

in ascorbic acid oxidase. Furthermore, it was demonstrated that approximately 30 % of the total ascorbic acid oxidase activity of the first five millimeters of the root tip is found in the soluble or supernatant fraction while the remainder of the activity was associated with the cell wall (Mertz 1960). In an attempt to gain a further understanding of the physiological processes associated with cell differentiation, experiments were set up to determine if there is a DPNH oxidizing system coupled with the oxidation of ascorbic acid by the soluble ascorbic acid oxidase.

Materials and Methods

A hybrid of *Zea mays* L., (The University of Texas numbers 854 × 857) was the source of root material. Seeds were soaked in tap water for 24 hours during which time they were vigorously aerated. They were then washed with a detergent (Tide) and thoroughly rinsed with tap water. This procedure resulted in eliminating virtually all contamination by microorganisms during the period of germination. The seeds were then rolled in a double layer of moist filter paper and placed in Berzelius beakers in a dark room at 22°C. To insure sufficient moisture during the course of germination another Berzelius beaker was placed over the first. After five days the primary roots were removed from the seedlings, thoroughly washed in double distilled water, and stored in a cold room (8°C.) in double distilled water until ready for use. The apical 5 millimeters of the primary root was used in all experiments of enzyme identification. For the determination of the relative distribution of enzymatic activity along the root axis, the rootcap was removed prior to cutting the root into six successive millimeter segments using Gillette thin razor blades held in a small jig.

Cell number determinations were made using a hemocytometer. Millimeter segments were placed in a solution consisting of one part 2 % nitric acid and two parts 5 % chromic acid and incubated 2—3 hours at 50—60°C. The segments were then broken up by stirring rapidly with a small stainless steel stirrer.

Since heavy metals, particularly cupric ions, readily oxidize ascorbic acid the utmost care was exercised in preparing the roots for DPNH oxidase determinations. All homogenizing media and buffers were prepared in water which had been triple distilled in a borosilicate still.

Cell free homogenates were obtained after grinding the root segments in 1×10^{-3} M EDTA (pH 5.8—6.0) from 3 to 5 minutes in a Ten Broeck glass homogenizer. All procedures were carried out in an ice bath.

The gross homogenates were centrifuged at $850 \times g$. for five minutes in a clinical centrifuge in a cold room at 8°C. to remove the cell wall debris. The supernatant fraction was further centrifuged in a Servall Superspeed Refrigerated Centrifuge at 0° to 2°C. for thirty minutes at $30,000 \times g$. The pellet from the $30,000 \times g$. spin was resuspended in the same volume of EDTA in which the root segments were originally homogenized. Although the pellet is probably composed of many intracellular components other than the mitochondria, the activity of this fraction is herein assumed to be largely due to the activity of the mitochondria.

Oxidation of DPNH was followed by measuring the decrease in optical density at 340 mμ in a Beckman DU Spectrophotometer using silica cells of 1 cm. cross

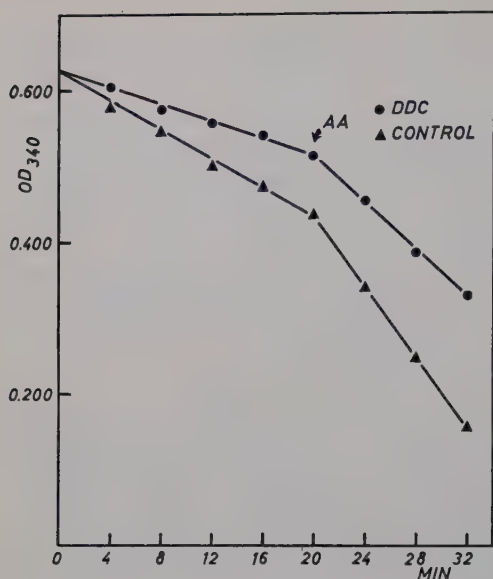


Figure 1. The effect of DDC upon the oxidation of DPNH by the supernatant from the first 5 mm. of the primary root in the presence of added ascorbate. 0.1 ml. 0.04 M ascorbate (AA) was added as indicated. Concentration of DDC in 3.1 ml. final volume was 5.0×10^{-4} M. The supernatant contained 62 micrograms N.

section. The cells usually contained 0.5 ml. 0.06 M phosphate buffer pH 7.0, 0.3 ml. enzyme preparation, 0.3 ml. approximately 0.3 mg.) DPNH, 0.1 ml. 0.04 M ascorbic acid and water to a total volume of 3.0 or 3.1 ml.

Nitrogen determinations were carried out according to the Nessler procedure of Umbreit *et al.* (1957).

Results

The addition of the supernatant to DPNH induced a rapid oxidation; however, upon heating the supernatant for 2 minutes in a water bath at $100^{\circ}\text{C}.$, complete loss of activity resulted. The rate of decrease of optical density at 340 m μ was linear with time. The addition of sufficient ascorbic acid to give a final concentration of 1.3×10^{-3} M (see control, Figure 1) resulted in an increase in the oxidation of DPNH by over 100 %. The addition of DHA had no effect upon the oxidation of DPNH, consequently it appears that some intermediate oxidation product of ascorbic acid is functioning as the electron acceptor. Figures 1 and 2 illustrate the effect of diethyldithiocarbamate (DDC) and potassium cyanide (KCN) upon the oxidation of DPNH in the presence of and in the absence of added ascorbic acid. 5×10^{-5} M DDC and 1×10^{-4} M KCN inhibits DPNH oxidation in the absence of added ascorbic acid by 41 and 25 % respectively. However, in the presence of added ascorbic acid the inhibitors have virtually no effect upon the increased oxidation caused by added ascorbic acid. Since it has been demonstrated manometrically that 5×10^{-5} M DDC results in virtually complete inhibition of ascorbic

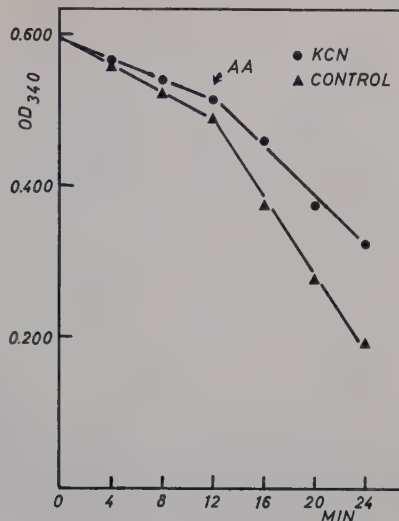


Figure 2. The effect of KCN upon the oxidation of DPNH by the supernatant from the first 5 mm. of the primary root in the presence of added ascorbate. 0.1 ml. 0.04 M ascorbate (AA) was added as indicated. Concentration of KCN in 3.1 ml. final volume was 1×10^{-4} M. The supernatant contained 53 micrograms N.

acid oxidation (Mertz 1960) and that KCN is also a powerful inhibitor of ascorbic acid oxidase, it may be concluded with a reasonable degree of certainty that the ascorbate-stimulated oxidation of DPNH is not solely due to the catalytic action of ascorbic acid oxidase. Furthermore, since the supernatant fractions were prepared in triple distilled water to which 1×10^{-3} M EDTA was added, the participation of free heavy metal catalysts may be also reasonably excluded.

Although the evidence just presented indicates that the ascorbate-stimulated oxidation of DPNH is not mediated by ascorbic acid oxidase, the possible participation of ascorbic acid oxidase cannot be entirely excluded since the oxidation of DPNH by the supernatant in the absence of added ascorbic acid is partially inhibited by DDC and KCN. Since both ascorbic acid and its oxidase are present in the root tip it would be reasonable to assume that at least a portion of the DPNH oxidation can be mediated by an endogenous ascorbic acid oxidase-ascorbate system.

Another important consideration concerning the oxidation of DPNH in the absence of added ascorbic acid is that it has been observed repeatedly that enzyme preparations left standing 3—4 hours at 8°C. lose their ability to oxidize DPNH by roughly 50 %, while the oxidation of DPNH in the presence of added ascorbic acid is unaffected. This loss of activity by the supernatant strongly suggests that the endogenous ascorbic acid present in the root tip at the time of homogenization is oxidized to DHA, and as such cannot participate in the oxidation of DPNH. Furthermore, since it was previously demonstrated that DHA had neither a stimulatory nor an inhibitory

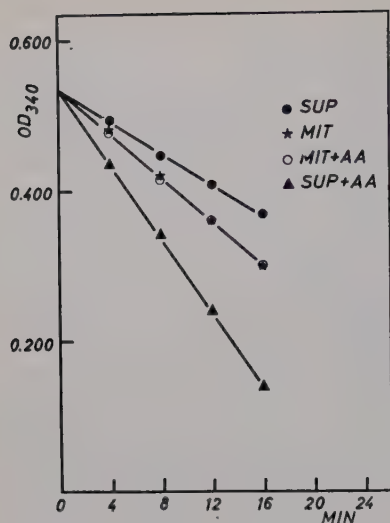


Figure 3. A comparison of DPNH oxidation by the supernatant (SUP) and particulate mitochondrial fraction (MIT) in the presence and in the absence of 0.1 ml. 0.04 M ascorbate (AA). The supernatant fraction contained 40 micrograms N, the mitochondrial fraction 22 micrograms N.

effect upon the oxidation of DPNH, the loss of activity is undoubtedly not induced by DHA acting as an oxidizing agent of reactive —SH groups, which may be an inherent property of the system responsible for the oxidation of DPNH (James 1953). Whether complete loss of DPNH oxidase activity would have resulted if the endogenous ascorbic acid had been entirely removed by dialysis or some other treatment remains to be investigated; however, there appears to be no question that the endogenous ascorbic acid plays an important role in the oxidation of DPNH, either through its oxidation by the DDC-KCN insensitive fraction or through its oxidation by ascorbic acid oxidase.

Figure 3 compares the supernatant activity with that of the mitochondrial fraction in the presence of and in the absence of added ascorbic acid. The mitochondrial fraction is roughly 30 % more active than the supernatant fraction; but ascorbic acid does not increase the oxidation of DPNH. This is evidence for the participation of a soluble electron transporting mechanism involving ascorbic acid which is separate and distinct from the particulate mitochondrial system.

As a possible means of correlating some function of the ascorbate stimulated oxidation of DPNH with cellular differentiation, six successive millimeter segments along the longitudinal axes of the root were assayed. Since analysis of successive segments along the root axis would be of limited value without reference to the overall growth pattern, the general regions of the maize root identified by Erickson and Goddard (1951) will be taken as reference points. They have demonstrated that approximately 0.5 mm. to 2.5 mm.

Table 1. *DPNH oxidation on a per cell per segment basis in the absence of and in the presence of added ascorbic acid. $OD_{340} \times 10^{-7}$ per 16 min. and cell.*

Segments	Control	Added ascorbate
Rootcap	8.0	40.0
0 — 1 mm.	3.8	5.0
1 — 2 "	4.6	6.3
2 — 3 "	5.7	10.3
3 — 4 "	5.0	11.6
4 — 5 "	4.7	10.6
5 — 6 "	4.6	12.2

from the apex is the region in which cell division is occurring at a high rate. Basipetally from this region cell extension proceeds at a relatively rapid rate, reaching a maximum between 4 mm. and 5 mm. from the apex. Since each successive segment cut basipetally from the apex contains fewer cells, but cells in progressively more advanced stages of development, the quantitative values obtained per segment were divided by the total number of cells in each segment to provide a series of values which would represent the changes that occur as cells expand and elongate.

Table 1 illustrates the relative distribution of DPNH oxidation on a per cell per segment basis in the absence of (control) and in the presence of added ascorbic acid. In the control, DPNH oxidation per cell is relatively low in the region of maximum cell division, the 0—1 mm. and 1—2 mm. segments. In segments 2—3 mm. and 3—4 mm., the region of rapid cell elongation, the oxidase activity shows a slight increase and then levels off. In the presence of added ascorbic acid DPNH oxidation is again rather low in the region of cell division; however, from this point basipetally there is a rather marked increase in DPNH oxidation along the entire axis of the root assayed. Although the decrease and subsequent leveling off of the oxidation of DPNH in the control, while the oxidation progressively increases in the presence of added ascorbic acid may be a reflection of a loss of the endogenous ascorbic acid during the homogenization procedures, thus limiting the oxidation of DPNH, the fact still remains that as cells from the meristematic zone undergo expansion and elongation there is a concomitant increase in the ability of the cells to oxidize DPNH.

The rootcap was also assayed for DPNH oxidase activity (Table 1). In the presence of added ascorbic acid the activity per cell increases by 400 % of the control, and is roughly three times as active as the most active cell of the 5—6 mm. segment. Although this rather striking increase in the ascorbate-stimulated oxidation of DPNH may again be a reflection of a loss of the endogenous ascorbic acid during the homogenization procedures it empha-

sizes the participation of ascorbic acid oxidase, for it has been previously demonstrated that the oxidase of the root cap is largely associated with the soluble or supernatant fraction, while in the root proper roughly 70 % of the total ascorbic acid oxidase activity is associated with the cell wall (Mertz 1960). Such an active oxidizing system in the rootcap may be a reflection of the developmental difference between the cells comprising the rootcap and the more basipetal segments of the root proper. The bulk of the rootcap cells may be regarded as relatively mature and as such represent a developmental state several stages removed from the relatively immature cells of the basal segments.

Discussion

Although it is not possible from the present study to ascertain how much respiratory H-transfer occurs through the DPNH- ascorbate system, it is noteworthy that the greatest ascorbate-stimulated oxidation of DPNH is associated with those cells which possess the greatest respiratory oxygen uptake. Goddard and Meeuse (1950) reported that the respiration on a nitrogen basis in roots of *Zea mays* is low in the meristematic zone, rises strongly and reaches a maximum in the extension zone and sinks to a constant level after the cessation of growth. Similar observations have been reported by Brown and Broodbent (1950) for bean roots and in barley roots by Eliasson (1955). Jensen (1955) has demonstrated in the roots of *Vicia faba* that the cells of the rootcap possess the highest oxygen uptake. This association certainly suggests that at least a portion of respiration can proceed through the DPNH-ascorbate system, and is not in keeping with Hackett's (1958) suggestion that the reduced pyridine nucleotide-ascorbic acid oxidase systems that have been described in various plant materials are "isolation artifacts" which have no physiological importance for respiration.

It will be recalled that the addition of ascorbic acid to the mitochondrial fraction had no stimulatory effect upon the oxidation of DPNH; therefore, it may be concluded that the DPNH-ascorbate system is not involved in the transfer of electrons in the energy-yielding reactions of mitochondrial respiration. Such an oxidative pathway which does not involve mitochondria, and is in part insensitive to DDC and KCN may be caused by a partial inhibition of one pathway of electron transport (ascorbic acid oxidase) and a resulting by-pass of electrons through a less sensitive system (the DDC-KCN insensitive system). The participation of such an alternate electron transporting mechanism may be one explanation for the cyanide and carbon monoxide resistant oxygen uptake which has been so frequently reported in the literature (Daly 1954, Hackett 1957, and Yocum and Hackett 1957).

Summary

A study has been made of the relationships among L-ascorbic acid, ascorbic acid oxidase, and DPNH oxidation. The evidence presented indicates that within the first few millimeters of the maize root tip there is a soluble electron transporting mechanism wherein ascorbic acid oxidase, and a potassium cyanide (KCN) -diethyldithiocarbamate (DDC) insensitive fraction are responsible for an oxidation product of ascorbic acid, which can serve as an intermediate electron acceptor between DPNH and molecular oxygen.

The investigation on which this report is based was a part of a dissertation submitted to the graduate school of the University of Texas in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

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The Relation of Iron Supply to the Tissue Concentration of Iron, Chlorophyll and Catalase in Barley Plants Grown in Sand Culture

By

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Introduction

In a previous paper, Sharma (19) had described the effect of iron supply on the growth and ascorbic acid content of barley plants grown in sand culture. It was shown that plants raised at 0.056 to 0.56 p.p.m. of iron supply showed characteristic visual symptoms of iron deficiency and had low yields. An iron supply of 0.70 p.p.m. was shown to be critical with respect to the general growth and yield of barley plants. The work described in this paper was further undertaken to find out the relation of iron supply to the tissue concentration of iron, a subject which deserved investigation in view of the reported anomaly of iron accumulation in plants showing true and induced iron deficiency. A study was also made of the iron porphyrin enzyme catalase and of chlorophyll, the synthesis of which may be dependent on iron supply, in order to gain an insight into the relationship between supply, uptake and physiological availability of iron.

Methods

The details of the culture methods used for raising barley (*Hordeum vulgare* var. K 12) plants at graded levels of iron supply ranging from 0.056 to 11.2 p.p.m. in sand culture and sampling have been described elsewhere (19). As in the earlier paper (19), the levels of iron supply ranging from 0.056 to 0.56 p.p.m. (both inclu-

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sive) are referred to as 'lower' levels and from 0.70 to 11.2 p.p.m. (both inclusive) as 'higher' levels.

The estimations of tissue iron, chlorophyll, and catalase were made at two stages of growth. At the first stage, 30 to 32 days growth, all plants irrespective of the level of iron supply had two to three leaves. The first leaf was apparently normal green in all the treatments but the other leaves of plants raised at lower levels of iron supply were chlorotic. The first and the other leaves were, therefore, separately estimated for tissue iron, chlorophyll and catalase. At the second stage, 60 to 62 days growth, samples of first leaf were not drawn and only two leaves, the youngest leaf and the one next to it, were estimated for iron, chlorophyll, and catalase. At the second stage of sampling, there were only 3 to 5 well developed leaves at the lower levels while at the higher levels the number of leaves varied from 5 to 6. The estimations of iron were made on oven dried leaf material and those of chlorophyll and catalase on fresh leaf lamina. Before drying, leaves for iron estimations were thoroughly washed to avoid any surface contamination.

Iron (total iron) was determined colorimetrically as the ferrous-orthophenanthroline complex (10) after wet digestion of the oven dried samples with nitric-perchloric acids (17).

Chlorophyll was estimated colorimetrically in acetone extracts by the method of Petering *et al.* (16). The calibration curve for chlorophyll was prepared by the method of Comar and Zscheile (5).

Catalase was determined in crude tissue extracts. Owing to the doubtful reliability of the permanganate and spectro-photometric methods for the estimation of catalase in crude tissue preparations (7), catalase was assayed manometrically by an adaptation of the method described by Chance and Maehly (4). The results are expressed as μ l of oxygen evolved by 10 mg. fresh tissue in 5 minutes time.

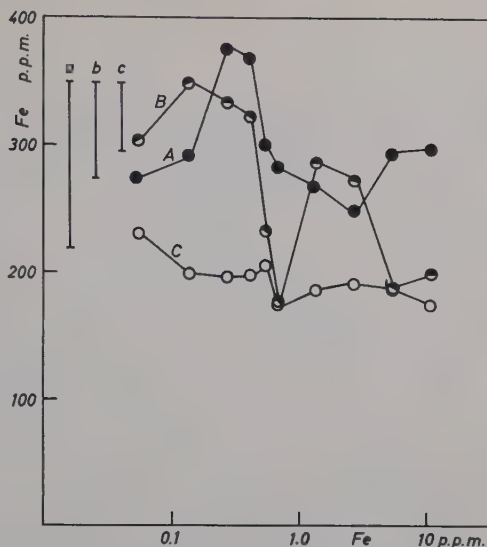
All estimations were carried out in duplicate and the data has been tested for significance at 5 % and 1 % probability levels.

Results

The relation of iron supply to tissue concentration of iron. The tissue concentration of iron did not bear any definite relation to the iron supply. With an increase in the iron supply, two hundred fold between the lowest and the highest, there was hardly any appreciable increase in the concentration of iron in leaves. On the other hand, severely chlorotic leaves of plants grown at low levels of iron supply showed a considerable accumulation of iron. At the first stage of growth the tissue concentration of iron in the leaves (other than the first) of plants grown at 0.056 to 0.42 p.p.m. iron supply was much higher than at any of the higher levels (Figure 1) and there was a negative correlation (statistically significant, $P < 0.05$) between iron supply and iron concentration (Table 1).

The relation of iron supply to chlorophyll. Iron supply had a marked effect (statistically significant, $P < 0.01$) on the chlorophyll content of the barley

Figure 1. *The effect of iron supply on tissue concentration of iron in barley plants grown in sand culture. A: Tissue concentrations of iron (in p.p.m. per dry matter) in the first leaf, 30 to 32 days growth. — B: Tissue concentration of iron in leaves other than the first, 30 to 32 days growth. — C: Tissue concentration of iron in young leaves, 60 to 62 days growth. — a, b and c denote the least difference for significance (L.S.D.) at $P=0.05$ for A, B and C respectively.*



leaves. Chlorophyll showed an increase with an increase in iron supply from 0.056 to 0.70 p.p.m. at the first stage and from 0.056 to 2.8 p.p.m. at the second stage. Above 0.70 and 2.8 p.p.m. iron supply at the first and the second stage of growth respectively chlorophyll concentration remained more or less unaffected. Compared to the first leaf, the effect of iron supply was more marked in the subsequent leaves. In the latter the increase in chlorophyll was very pronounced between 0.42 and 0.70 p.p.m. iron supply (Figure 2).

The relation of iron supply to catalase. The effect of iron supply on catalase in the first leaf of plants, at 30–32 days growth, was not marked. But

Table 1. *The correlations between iron supply and (i) tissue concentrations of iron (ii) chlorophyll and (iii) catalase and between (iv) chlorophyll and catalase in barley plants grown at graded levels of iron supply in sand culture. — The values of correlation coefficients (r) marked with * are significant at $P=0.05$ and with ** significant at $P=0.01$.*

Factors	Correlation coefficient (r)		
	First stage of growth		Second stage of growth
	First leaf	Other leaves	Young leaves
(i) Iron supply \times tissue concentration of iron	+ 0.097	– 0.456*	– 0.387
(ii) Iron supply \times chlorophyll	+ 0.506**	+ 0.570**	+ 0.610**
(iii) Iron supply \times catalase	+ 0.255	+ 0.611**	+ 0.526*
(iv) Chlorophyll \times catalase	+ 0.629**	+ 0.971**	+ 0.972**

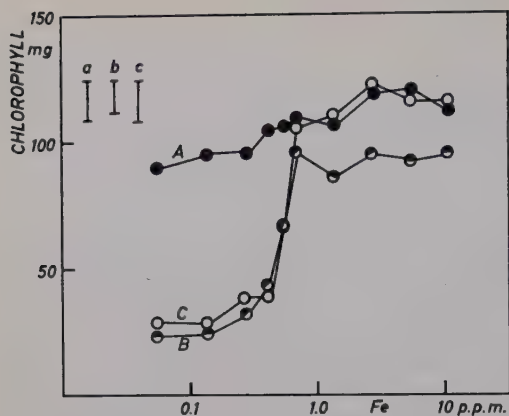


Figure 2. The effect of iron supply on the chlorophyll content of barley plants grown in sand culture. A: Chlorophyll content in mg./100 g. fresh tissue of the first leaf, 30 to 32 days growth. — B: Chlorophyll content of the leaves other than the first, 30 to 32 days growth. — C: Chlorophyll content of the young leaves, 60 to 62 days growth. — a, b and c denote the least difference for significance (L.S.D.) at $P=0.05$ for A, B and C respectively.

in the other leaves, at both the stages of growth, catalase was markedly affected (statistically significant, $P < 0.01$) by the iron supply. With an increase in iron supply from 0.056 to 0.70 p.p.m. catalase showed a marked increase (about three fold). Above 0.70 p.p.m. catalase was generally not appreciably affected by the iron supply. At both the stages of growth catalase at the lower levels of iron supply was significantly lower than at higher levels of iron supply (Figure 3).

The chlorophyll-catalase relationship. The chlorophyll and catalase in barley leaves showed a close parallelism with respect to iron supply (Figure 4). In the first leaf, both chlorophyll and catalase were only feebly affected (though statistically significant in case of chlorophyll) by the iron supply. But in the leaves other than the first, at the two stages of growth, iron supply

Figure 3. The effect of iron supply on catalase in barley plants grown in sand culture. A: Catalase in the first leaf, 30 to 32 days growth. — B: Catalase in the leaves other than the first, 30 to 32 days growth. — C: Catalase in young leaves, 60 to 62 days growth. — Catalase expressed as $\mu\text{l. O}_2$ evolved by 10 mg. fresh tissue in 5 min. — a, b and c denote the least difference for significance (L.S.D.) at $P=0.05$ for A, B and C respectively.

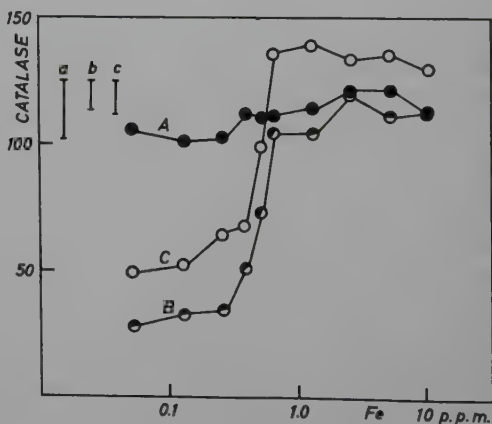
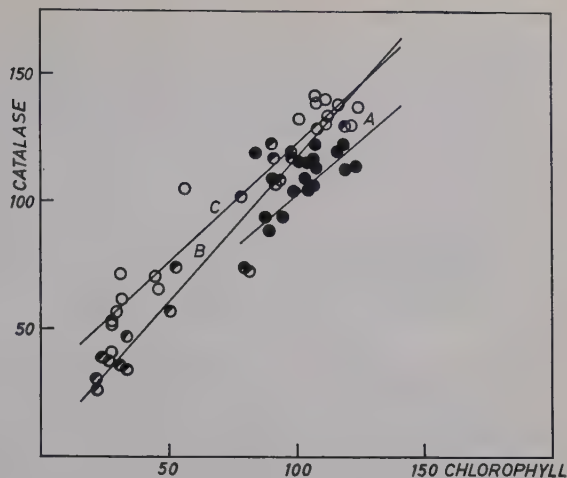


Figure 4. *The relationship between chlorophyll and catalase in barley plants grown in sand culture. A: First leaf, 30 to 32 days growth. — B: Leaves other than the first, 30 to 32 days growth. — C: Young leaves, 60 to 62 days growth. Chlorophyll and catalase measured as in Figures 2 and 3. — For A, $Y=43.5+0.646 X$; B, $Y=3.6+1.136 X$; and C, $Y=29.9+0.926 X$.*



brought about a very marked effect (statistically highly significant, $P < 0.01$) in both chlorophyll and catalase. When iron supply was less than 0.70 p.p.m., a concentration shown to be critical for the growth of barley, both chlorophyll and catalase were markedly depressed. With the iron supply expressed logarithmically and chlorophyll or catalase arithmetically the curves for both chlorophyll and catalase in the barley leaves (not the first leaf) were roughly sigmoid with a sharp zone of inflection between 0.28 p.p.m. and 0.70 p.p.m. iron supply at the first stage of growth and between 0.42 p.p.m. and 0.70 p.p.m. iron supply at the second stage of growth (Figures 2 and 3). Between 0.70 p.p.m. and 11.2 p.p.m. iron supply both chlorophyll and catalase were near optimal at both the stages of growth. As is evident from Table 1, there was a highly significant positive correlation between chlorophyll and catalase in relation to iron supply.

Discussion

Literature pertaining to the tissue concentration of iron in plants raised at known levels of iron supply in sand or water culture is very scanty. In this respect whatever information is available has been derived from the experiments primarily carried out to study the metal interrelationships in plants. Some of the more relevant data from metal interrelationship experiments of the different workers are given below.

Table 2 clearly shows that the concentration of iron does not always increase with an increase in iron supply from a low to a normal or high level.

Table 2. *The tissue concentration of iron in plants grown at graded levels of iron supply.* Data taken from Table 3 of Somers and Shive (21), Table 4 of Jones and Hewitt (13), Tables 16 and 17 of Twyman (22), and Table 1 of Weinstein and Robbins (24). — Data of Somers and Shive, Twyman and Weinstein and Robbins are obtained from water culture experiments and those of Jones and Hewitt from sand culture experiments. At levels of iron supply marked with * plants showed characteristic symptoms of iron deficiency.

Investigators	Plant species	Iron supply in nutrient medium p.p.m.	Iron concentration in plant tissue p.p.m. dry matter
Somers and Shive	Soyabeans (tops)	0.005*	213.5
		0.50	297.4
		3.00	344.0
Jones and Hewitt	Potato (young leaves)	0.0056*	194
		5.6	135
		28.0	171
Twyman 1944 Experiment	Oats (tops)	0.005*	68
		0.50	77
		3.00	83
	" "	0.005*	255
		0.01	193
		0.50	91
		3.00	126
Weinstein and Robbins ...	Sunflower (leaves)	0.001*	76.5
		0.25	169.5
		10.00	191.0

From his data Twyman (22) concluded "Increasing the iron supply may cause an increase in the iron concentration in the tissues . . . But this is not always the case . . . When iron is in low concentration in the medium it may accumulate in the tissues to very high levels (*e.g.*, 368 p.p.m. Table 17), and bear no relation at all to the concentration in the substrate".

We did not find any consistent correspondence, whatsoever, between iron supply and the tissue concentration of iron in barley. The tissue concentration of iron in plants showing luxuriant growth was in no way consistently higher than in plants exhibiting typical symptoms of iron deficiency (raised at low levels of iron supply). On the other hand, there was some accumulation of tissue iron at some of the lower levels. Our results clearly show that in barley there is no linear (or curvilinear) relationship between iron supply (expressed logarithmically) and the iron concentration in the tissue as discussed by Hewitt (9) and that in the case of iron nutrition of this plant no 'critical percentage' or 'critical nutrient concentration' as suggested by Macy (14) and Ulrich (23) can be made out.

In spite of appreciable amount of iron in the tissues, plants grown at low levels (0.056 to 0.56 p.p.m.) of iron supply showed effects characteristic of

the deficiency of iron. Thus suggesting that all the iron was not in the physiologically active form. This is in accord with the suggestion of Oserkowsky (15), Somers and Shive (21), Jacobson (11), Bennett (2), Hewitt (8) and Twyman (22) that all the iron in the chlorotic tissue is not in an 'active', or available form.

We found that in barley catalase and chlorophyll were, each, significantly positively correlated with iron supply. These results are in agreement with the earlier findings regarding the relation of iron supply to chlorophyll in pineapple (20) and sunflower (12) and to catalase in sunflower (24) and soyabeans (1).

A fairly close parallelism between chlorophyll and catalase in relation to iron supply would suggest that iron supply determines the synthesis of some precursor of both chlorophyll and catalase. Granick (6) has suggested that protoporphyrin — 9 is a common precursor of chlorophyll and the haem part of catalase. This type of compound has also been suggested to be concerned in the uptake of iron (Robertson, 18). Iron combined with a porphyrin is physiologically active and if the total uptake of iron in plants were to take place by combination with an acceptor, say "*acceptor-X*" which is a porphyrin, plants would not show an accumulation of iron in the tissues and at the same time effects characteristic of iron deficiency. *It, therefore, appears that plants take up iron in more than one way; iron which is taken up by combination with acceptor-X, which is either protoporphyrin-9 or one of its precursors or a compound which regulates its synthesis, is the physiologically active iron. Iron taken up without combination with acceptor-X is not available for the different metabolic processes.* The results obtained by us, namely, depression in chlorophyll and catalase and accumulation of iron in tissues of plants raised at low levels of iron supply could be explained only if the iron supply also determines the synthesis of *acceptor-X* which would then correspond to the acceptor-A (not necessarily convertible to B) of Twyman (22) and the organic chelating compounds of iron suggested by Brown *et al.* (3).

Summary

1. The relation of iron supply to its tissue concentration, chlorophyll and catalase in barley plants raised at graded levels of iron supply, ranging from 0.056 to 11.2 p.p.m. in sand culture has been studied at two stages of growth.
2. The iron supply did not bear any definite relation to the tissue concentration of iron.

3. The chlorophyll content of the leaves was significantly positively correlated with iron supply.
4. Catalase in leaves was positively correlated with iron supply. The correlation between iron supply and catalase in leaves other than the first was found to be statistically significant.
5. In relation to iron supply, there was a marked parallelism between chlorophyll and catalase in leaves of barley plants and the two tissue components were found to be highly significantly ($P < 0.01$) positively correlated.
6. It has been suggested that iron supply determines the synthesis of some porphyrin-like acceptor of iron, "*acceptor-X*", combined with which alone is iron physiologically active or available, and that *acceptor-X* is a precursor of, or is identical with protoporphyrin-9, a common precursor of the catalase haem and the chlorophyll.
7. The accumulation of iron in plants showing characteristic effects of iron deficiency has been explained by further suggesting that uptake of iron takes place in more than one way. It is only the iron combined with *acceptor-X*, which is physiologically active or available.

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Dosage du zinc chez un lichen calaminaire belge: *Stereocaulon nanodes* Tuck. f. *tyroliense* (Nyl.) M. Lamb

Par

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Travaux lichénologiques V

(Reçu le 5 Decembre, 1960)

Au cours d'un travail précédent (1), deux d'entre nous ont présenté quelques considérations sur la teneur en zinc des plantes des terrains calaminaires caractéristiques du *Violetum calaminariae* en Belgique.

De ces observations, il résulte que la majorité des plantes de ce biotope non seulement absorbent le zinc mais que certaines, telles *Thlaspi alpestre* var. *calaminare* et *Armeria maritima* var. *Halleri*, sont capables de l'accumuler dans les rhizomes ou les parties aériennes.

La question se pose immédiatement de savoir pourquoi les plantes de cette association sont capables de tolérer de telles teneurs en zinc et sous quelle forme le zinc est fixé dans les tissus. Nous n'avons pas encore entrepris de répondre à ces questions et, avant de quitter le plan de l'observation, nous avons voulu savoir comment certaines thallophytes, un lichen en l'occurrence, réagissait vis-à-vis du zinc. Ce lichen est un *Stereocaulon*, strictement limité en Belgique aux terrains calaminaires de l'est du pays. Il s'agit d'une espèce alpine qui, ici, a trouvé place au sein de la communauté du *Violetum calaminariae* sans qu'il soit actuellement possible d'en expliquer la raison. La présence dans ce biotope d'un lichen du genre *Stereocaulon* a été signalée jadis par P. Duvigneaud, mais c'est l'un d'entre nous (I. M. Lamb) qui a pu établir son identité exacte. Il s'agit exactement de *Stereocaulon tyroliense* (Nyl.) Lett. (1918), qui est en fait un taxon infraspécifique se rattachant à *St. nanodes* Tuck. dont il représente une forme "in which true podetia are mostly absent, and the thallus consists of flattened-bifacial squamules directly

sessile on the rock". Une nouvelle combinaison doit codifier cette vue dans le cadre de la nomenclature:

Stereocaulon nanodes Tuck f. *tyroliense* (Nyl.) M. Lamb, comb. nov.

Basionyme: *Stereocladium tyroliense* Nyl., in *Flora*, LVIII, p. 302, 1875.

Le *Stereocaulon* utilisé dans cette note a été récolté dans deux de ses stations, celle d'Angleur, près de Liège, entre l'Ourthe et le canal du même nom, et celle du Rocheux, à Oneux près de Theux.

Stereocaulon se trouve à la fois sur des scories d'une exploitation ancienne, sur des moules de texture granuleuse et d'aspect gréseux et sur des briques ayant probablement servi à certaines constructions en rapport avec le traitement du minerai.

Le dosage du zinc a été effectué par la méthode polarographique: celle-ci se prête très bien à la détermination du zinc dans les végétaux et dans les sols et divers auteurs l'ont appliquée dans ce but. Il est toutefois indispensable de faire précéder la détermination polarographique de certaines manipulations chimiques destinées à éliminer de la solution des métaux gênants.

Stout, Levy et Williams (3) réalisent cette séparation par la méthode à la dithizone.

Reed et Cummings (2) simplifient le procédé en éliminant la majeure partie des éléments gênants par précipitation à l'ammoniaque, à un pH contrôlé. Le mode opératoire que nous avons adopté précipite une partie des métaux présents sous forme de sulfures, les autres sous forme d'hydroxydes, et la solution qu'on soumet à l'analyse polarographique ne contient pratiquement plus que le zinc, le calcium, le magnésium et les métaux alcalins.

On incinère 1 gr. de matière sèche à température aussi basse que possible et, en tous cas, inférieure à 450°C, pour éviter les pertes de zinc par volatilisation. Les cendres sont attaquées par 2 à 3 ml de HCl 6 N; on évapore ensuite à siccité. On reprend le résidu par 0,5 ml de HCl concentré et on additionne, après quelques instants, de 5,5 ml d'eau chaude. La solution ainsi obtenue est traitée, jusqu'à saturation, par un courant d'acide sulfhydrique; au cours de cette opération, on dilue à trois reprises, par 3 ml d'eau.

L'insoluble et le précipité des sulfures sont séparés par filtration et lavés. A côté du zinc, il y a dans le filtrat du fer, de l'aluminium, du manganèse, du calcium, du magnésium et les alcalins. On le porte à ébullition pour éliminer H₂S, on ajoute quelques gouttes de HNO₃ 6 N pour réoxyder le fer, puis on évapore à sec au bain-marie. Le résidu est repris par 0,5 ml de HCl 12 N et quelques cc d'eau et transvasé quantitativement dans un ballon jaugé de 50 ml. On introduit encore dans ce dernier 2 à 3 ml d'eau oxygénée et un excès d'ammoniaque (5 à 6 ml de NH₄OH 6 N); on abandonne au repos pendant plusieurs heures.

Après avoir complété au volume par de l'eau, on fait une filtration partielle pour séparer le précipité de $\text{Fe}(\text{OH})_3$, $\text{Al}(\text{OH})_3$, $\text{MnO}(\text{OH})_2$. On évapore à sec 10 ml du filtrat, reprend par 25 ml de la solution de base et on procède à l'enregistrement polarographique.

Dans les conditions décrites ci-dessus, on obtient pour le zinc une vague parfaitement définie, où n'apparaît pas la moindre perturbation.

Le polarographe utilisé est le SARGENT Mod. XXI.

Les polarogrammes ont été enregistrés entre $-0,7$ et $-1,7$ volt, à la température de 22°C ($\pm 0,5^\circ$), le temps de goutte étant de 4 secondes.

La solution de base employée est celle préconisée par Stout, Levy et Williams (3), c'est-à-dire une solution $0,1\text{ N}$ en acétate ammoniacque et $0,025\text{ N}$ en sulfocyanate potassique. Le potentiel de demi-vague du zinc se situe dans ces conditions à $-1,05\text{ V}$.

Le calcul des résultats se fait au moyen d'une courbe d'étalonnage établie avec des solutions de concentrations connues en zinc.

A titre comparatif et en guise de témoin, nous avons également recherché le zinc dans une autre espèce du même genre, étrangère aux terrains calaminaires, *Stereocaulon vesuvianum* Pers. var. *denudatum* (Flk.) M. Lamb f. *umbonatum* (Wallr.) M. Lamb, récolté sur des éboulis pierreux (quartzites) dans la vallée de la Warche.

Avant d'être réduits en poudre, les échantillons de lichens sont soit lavés par un jet d'eau distillée, soit totalement plongés dans celle-ci, puis égouttés sur filtre. Cette préparation du matériel s'impose car, parmi les thalles, de la poussière contenant du zinc s'accumule et les dosages réalisés perdent leur signification. Nous donnons d'ailleurs pour un même échantillon, dans le tableau 1, le pourcentage en zinc obtenu avant et après lavage et justifions ainsi la nécessité de ce prétraitement.

Tableau 1. Dosage du zinc dans *S. nanodes* f. *tyroliense* non lavé et lavé à l'eau distillée.

	Stereocaulon nanodes non lavé	Stereocaulon nanodes lavé
Matière sèche	0,500 gr	0,250 gr
Cendres	0,220 gr	0,094 gr
Zinc en % de matière sèche	0,79 % — 7900 p.p.m.	0,57 % — 5700 p.p.m.

Le lavage a été réalisé au maximum en plaçant le lichen sur un filtre et en l'arrosant à plusieurs reprises avec de l'eau distillée. Cette expérience préliminaire ne laisse aucun doute sur la nécessité de laver les lichens avant dosage du zinc.

Dans une première série de dosage, nous avons, sur des lichens lavés par

jet d'eau distillée, obtenu les résultats suivants pour le site d'Angleur. Les dosages portent sur lichens récoltés à la fois sur des scories, des moules et des briques, ainsi que sur ces substrats eux-même.

Tableau 2. Zinc en ‰ de matière sèche. — Lichen et substrat. Angleur.

	Brique	Lichen	Scorie	Lichen	Moule	Lichen
Matière sèche	1 gr	0,500 gr	1 gr	0,500 gr	1 gr	0,500 gr
Cendres	0,989 gr	0,1337 gr	0,993 gr	0,1559 gr	0,9836 gr	0,142 gr
% Zinc	0,72	0,75	0,027	0,83	1,16	0,88
Matière sèche			1 gr		1 gr	
Cendres			0,93 gr		0,98 gr	
% Zinc			0,52		1,87	
Matière sèche			1 gr			
Cendres			0,980 gr			
% Zinc			0,9			

Une seconde série de dosages a été ensuite réalisée dans *S. nanodes* provenant d'une part d'Angleur et d'autre part du site d'Oneux. Cette fois les échantillons se trouvaient sur des scories (trash) et ont été lavés par un courant d'eau distillée.

Le tableau III fait part des résultats; le zinc est exprimé en ‰ de matière sèche.

Tableau 3. Dosage du zinc dans des échantillons lavés par un courant d'eau.

	Angleur		Oneux	
	Scorie	Lichen	Scorie	Lichen
Matière sèche	1 gr	0,6658	1 gr	1 gr
Cendres	0,937 gr	0,2318 gr	0,971 gr	0,507 gr
% Zinc	0,52	0,53	0,07	0,33

A titre documentaire et comparatif, nous donnons les résultats obtenus avec un *Stereocaulon* étranger aux terrains calaminaires, *S. vesuvianum* prélevé sur quartzite dans la vallée de la Warche.

Tableau 4. Pourcentage de zinc dans *S. vesuvianum*, espèce non calaminaire.

	Quartzite	Lichen
Matière sèche ...	1 gr	1 gr
Cendres	0,971 gr	0,087 gr
% Zinc	0,01	0,0225

De l'ensemble des analyses, on peut dégager les points suivants:

1. Sur terrains calaminaires, *S. nanodes* absorbe le zinc et, dans certains cas, celui-ci s'accumule d'une manière nette (site d'Oneux). En effet dans une scorie qui contient 700 p.p.m. de zinc, le lichen qui l'utilise comme substrat en renferme 3300 p.p.m. Quant au témoin non calaminaire, bien que les teneurs soient beaucoup plus faibles, elles donnent lieu aux mêmes constatations: 100 p.p.m. pour le substrat, 225 p.p.m. pour le lichen.

2. Le lavage préliminaire des échantillons a une grande importance comme l'indique les résultats du tableau I. En effet, dans les thalles de lichen s'accumulent par le jeu des facteurs environnants des poussières riches en zinc.

3. Le dosage du zinc dans plusieurs échantillons de scories montre que ces dernières ont des teneurs en zinc extrêmement variables. Quoiqu'il en soit les lichens qui les utilisent comme substrat ont très souvent des teneurs en zinc très voisines.

En conclusion, *S. nanodes* f. *tyroliense*, tout comme les plantes supérieures du "*Violetum calaminariae*" en Belgique, est capable d'absorber, d'accumuler et de tolérer des teneurs en zinc à première vue incompatibles avec le déroulement normal des processus métaboliques.

Un fait particulièrement curieux est que, chez nous, *S. nanodes* soit strictement limité, "on trash from old zinc mine", alors qu'en dehors il soit un organisme assez commun dans la région alpine. Le zinc le préserverait-il chez nous d'une certaine concurrence ou cet élément lui serait-il à ce point nécessaire? Il semble que l'on puisse déjà répondre à la dernière hypothèse en écartant la nécessité du zinc en tant que telle puisque ce lichen peut vivre dans des régions alpines a priori moins riches en zinc que les scories des "old zinc mines".

Summary

It has been shown that *Stereocaulon nanodes* Tuck. f. *tyroliense* (Nyl.) M. Lamb, a species confined in Belgium to calamine bearing soils takes up zinc and in some cases accumulates it in the thallus. This lichen using as a substrate slag with 700 p.p.m. of zinc may contain upto 3300 p.p.m. Zn.

This abnormally high content as compared with the usual plant tolerance for this element does not seem to disturb the metabolism. It is necessary to wash the samples in a stream of distilled water before extracting since zinc containing cinders can contaminate the thallae.

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Certain Comparative Studies on Physiological and Synergistic Actions of Gibberellin, with Indole- and Naphthalene Acetic Acids

By

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Introduction

For many years scientists have tried, from different angles, to compare the physiological actions of gibberellin (GB) with those of auxins. Various investigators (Yabuta and Hayashi 1939, Kato 1953, Brian and Hemming 1955 a) after performing comparative studies on gibberellin with other auxins, such as indole acetic acid (IAA), arrived at a common decision that the gibberellins could not be considered as auxins in the usual term of the word. Following these early findings the question whether gibberellin was an auxin-synergist attracted the attention of several research workers (Nitsch and Nitsch 1956, Brian *et al.* 1957, Kato 1958, Galston 1959), but not very clear conclusive results were obtained.

Concerning the physiological actions of GB, in the literature, very little attention was paid to the changes occurring in the water uptake, and its responsible factors, suction potential, tensility, and water-permeability. In this laboratory large series of experiments on this field were carried out with certain growth regulators possessing different nuclei, and satisfactory results obtained (cf. Hasman 1957, Hasman-Inanç 1958). Hence, to elucidate whether gibberellin is an auxin and produces similar reactions, in the first series of the present research it was decided to examine the GB action on the above mentioned uptake process and its factors with the technique used in this laboratory (cf. Hasman 1953 and 1957). The second aim of this paper

is to clarify the debatable question, in the relevant literature, of auxin-synergism of gibberellin. Therefore, an attempt was made to perform similar experiments using the same technique to verify the possible changes occurring in the water uptake, and its above cited factors under the combined action of gibberellin with IAA as well as naphthalene acetic acid (NAA).

Experimental Part

Material and method

For the main determinations of the water exchange between tissue and ambient medium the gravimetric immersion method was used. As the details of the technique was thoroughly explained in several former papers done in this laboratory (Hasman 1953, 1954, 1957), only the points where more elucidation was necessary were touched upon. As a suitable experimental object the parenchyma of potato tubers (*Solanum tuberosum*) was chosen, owing to the homogeneity and the negligible volume of its intercellular spaces (Boswell and Whiting 1940).

Preparation of the material

In an axial direction cylinders of several cm. in length were punched out from tubers of potato, using a cork borer of 8 mm. diameter. These cylinders were sectioned to discs of 5 mm. thickness with a razor blade and a brass pattern of 8 mm. bore and 5 mm. height. These dimensions were chosen because the percentage of dead or moribund cells in thicker sections is less than those in thinner ones (cf. Hasman 1957 b). To remove the contents of the injured cells at the wounded surface, the pieces were pretreated under running tap-water for 15 minutes and then rinsed several times in distilled water. The discs were then carefully blotted with soft filter paper.

After this procedure, the samples were divided into equal lots of 50 pieces each, for both auxin- and auxin synergism measurements. They were weighed in closed weighing glasses on an analytical balance, then were transferred into 250 ml. Erlenmeyer flasks of "Jenaer Geräte Glas G 20" containing 150 ml. of 10^{-5} M equimolar solutions of GB, IAA, and NAA for auxin measurements, as well as their combined isomolecular solutions of IAA-GB and NAA-GB respectively for auxin synergism determinations. As a control set 150 ml. of pure distilled water was used. The containers were well aerated by an electric compressor as described in several previous papers (cf. Hasman 1953, 1954, 1957), and were kept in a dark thermostat adjusted at 25.0°C. Measurements were taken at the 3rd, 6th, 24th, 48th hour. At the end of each of these exposure times the samples were blotted, weighed again, and the percentage increase in their fresh weight calculated. The suction potential determinations and the relative water permeability measurements were carried out as described in Hasman 1954, 1958. The calculations were made according to the formula given in the above papers.

Results

As cited in the introduction, this paper will deal firstly with the comparative auxin action of gibberellin on the water absorption and its responsible factors, the suction potential, tensility and water permeability, and secondly with the interaction of GB with IAA and NAA on the same processes.

Water Uptake

Although the effect of gibberellin on the water uptake, over long periods, has been touched upon in the relevant literature (Brian *et al.* 1955 b), the experiments were not carried out under very convincing conditions. Apart from this the action of GB upon a similar process, over short periods of time, was not enlightened.

In order to see whether GB exerts any auxin action on the water uptake process over short periods of time (3 to 48 hours), determinations were carried out using the gravimetric immersion method, and the effect of GB in the characteristic concentration of 10^{-5} M, was compared with the already known reactions of IAA and NAA, over the above mentioned period of time (Hasman-Inanç 1958).

The relative water uptake values (Q) in GB, IAA and NAA are graphically summarized in Figure 1.

As seen from Figure 1, the action of GB upon the water uptake process started within a period of 3 hours, slowly and steadily increased up to the 6th hour; towards the 24th hour it showed a tendency to slow down, thus indicating a decrease in the water uptake of the tissue. From this point onwards the graph still dropped slightly up to the 36th hour, henceforth followed a more or less linear course up to the 48th hour.

The IAA- and GB-curves, followed almost the same path up to the 3rd hour, within the 3 hour period leading to the 6th hour, the two curves gradually started separating. The effect of GB on the water uptake was pronouncedly less in comparison to that of NAA than IAA, as the NAA-curve followed a much steeper ascent up to the 6th hour.

Summing up the above results, it is permissible to conclude that, in general, GB produces much less effect on the water uptake process than IAA and NAA does, but up to 3 hours it shows an effect similar to that of IAA. Although the GB-curve diverges from the IAA-curve, between the 3rd and 6th hour interval, the values obtained are statistically insignificant.

After having observed the similarity of the IAA and the GB effect upon the water uptake process up to the 6th hour, it was decided to analyse the debatable synergistic action of the above mentioned compounds.

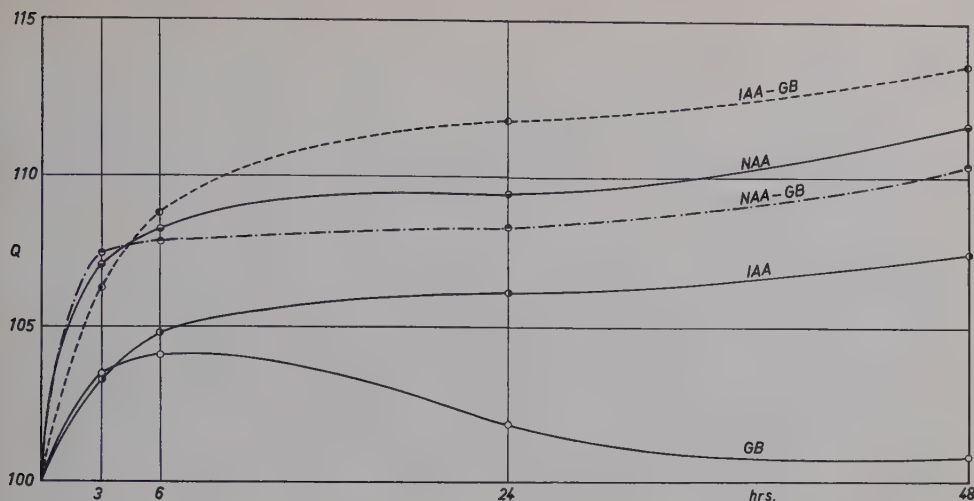


Figure 1. The relative water uptake (Q) determinations in GB, IAA, NAA and IAA-GB, NAA-GB. Abscissa: Time in hours.

$$\text{Ordinate: } Q = 100 \times \frac{\text{water uptake in compound}}{\text{water uptake in H}_2\text{O}}$$

A representative set of results are illustrated in Figure 1.

As Figure 1 demonstrates the IAA-GB curve shows a very steep ascent up to the 3rd hour, and it follows quite a rapid increase towards the 6th hour. Then the path of the curve continues with a diminished velocity until the 12th hour. Henceforth, the graph follows an almost linear course. As known in the literature, NAA produces a drastic effect on the water uptake of plant parenchyma in comparison to that of IAA. In the present case, the additive action of GB upon IAA by far exceeds that of the highly promoting reaction of NAA on the uptake process.

After obtaining the strong additive effect of IAA-GB, it was thought interesting to see whether the action of GB upon NAA would produce a promoting or retarding effect compared to the known action of NAA. The comparative results are put together with the previous values obtained (cf. Figure 1). As seen from Figure 1, the general trend of the curve is similar to that of pure NAA, but the reaction is slightly less pronounced; the values obtained are statistically significant only for the 24th and 48th hour.

From the results of these series the following conclusions can be drawn:

- 1) the gibberellin exerts a synergistic effect upon the water uptake process when added to IAA, according to the statistical calculations the additive action of IAA-GB is highly significant within the whole experimental period;
- 2) on the other hand, from the values obtained, it can also be deduced that

NAA-GB has a decidedly retardative action on the uptake process in comparison with the NAA-curve.

After having obtained, under the action of the applied compounds, the results of this chapter, it was thought suitable to examine the following primary factors which control the water exchange of the cells: first the suction potential (S.P.) and the tensility, and secondly the water permeability ($P_{w_{en}}$).

1. *Suction potential.* — In the first series of this chapter the suction potential (S.P.) of the potato tuber tissue, under the action of GB, was determined by the gravimetric immersion method. The changes were calculated as percentages of the original fresh weight, and were plotted against atmospheric equivalents of the corresponding gradations. The intersection points of the curves thus found, were compared with the corresponding points, obtained with the same technique, of the IAA-, NAA-curves. The results are summarized in Figure 2.

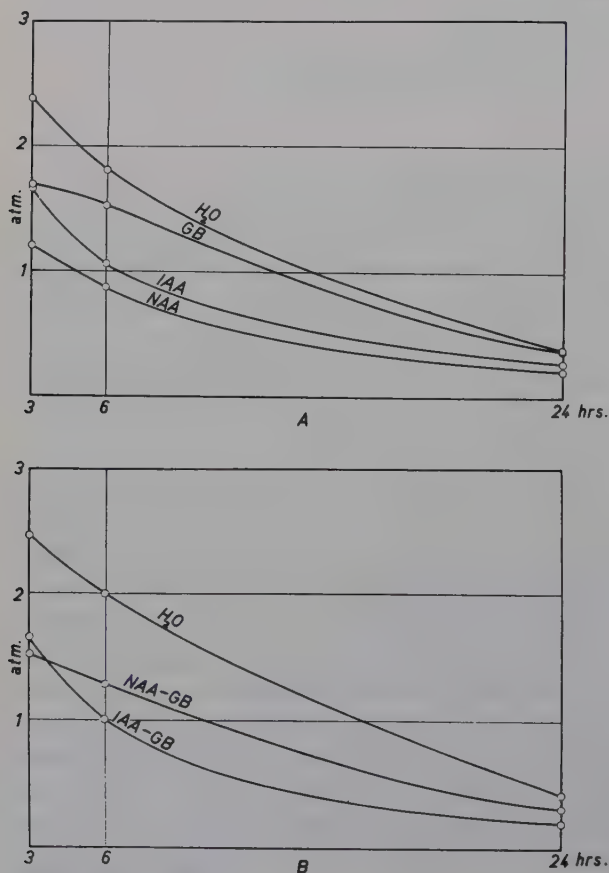


Figure 2. The comparison of intersection point determinations. A. single effect of compounds. B. synergistic effect of compounds. Abscissa: Atmosphere. Ordinate: Time in hours.

As seen from Figure 2 A, the intersection point-curves follow a parallel course in accordance with the water uptake graphs. Before drawing a final conclusion from the results of the latter series, as in the water uptake measurements, the corresponding determinations were carried out with the samples pretreated in IAA-GB, and NAA-GB. These results are shown in Figure 2 B. Here again the curves are in good agreement with the measured water uptake values (cf. Figure 1).

From the comparison of the intersection points of the S.P.-curves (Figure 3), it can be deduced that, GB, like the other auxins, seems to have no effect on the osmotic values of the cells, and that the diminishment in the S.P.-values under the influence of the compounds, is due to the increase in surplus water absorption. As noticed before, the degree of the water uptake in IAA combined with GB is more pronounced than that of NAA-GB, hence the concerned S.P.-values are in accordance with the above fact.

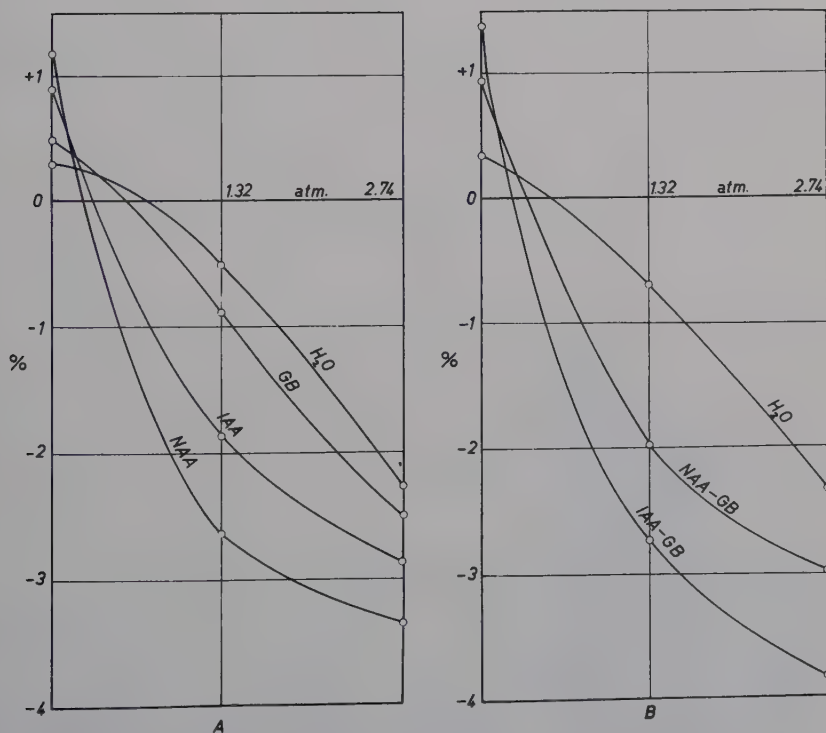


Figure 3. Suction potential determinations after 24 hours exposure time. A. single effect of compounds. B. synergistic effect of compounds. Abscissa: Atmosphere. Ordinate: Percentage changes in weight.

2. *Tensility*. — Although it was observed that GB had no effect on the S.P. of the cells, yet the trend of the obtained suction potential-graphs of the GB treated material, for later periods, showed the greatest divergence from the linear course in comparison with that of IAA and NAA which showed the least (Figure 3). According to previous findings of Hasman (1943) the degree of divergence of the positive part of the S.P.-curves from the linear course can be attributed to the limited tensility of the cell wall which controls the water uptake. Though GB limits the tensility of the cell wall, GB added to IAA increases even more the known effect of IAA on the tensility. These findings explain the course of the water uptake-curve, at the later periods, under the sole action of GB, and the synergistic effect of IAA-GB on the same process.

3. *Entrance water permeability*. — During the water uptake determinations, the uptake effect produced by GB as well as the other auxins, and the striking interaction of GB with IAA and NAA was noticed especially at the early hours, namely from the 3rd to the 6th. Having seen the rôle of the two responsible factors on the water absorption process under the sole- and synergistic effect of GB, it was thought suitable to analyse the third factor responsible at the early hours, namely the entrance water permeability (Pw_{en}).

For direct measurements of Pw_{en} the following formula was used:

$$Pw_{en} = \frac{\text{water exchange as \%}}{1/2 (\text{initial S.P.} + \text{final S.P.})}$$

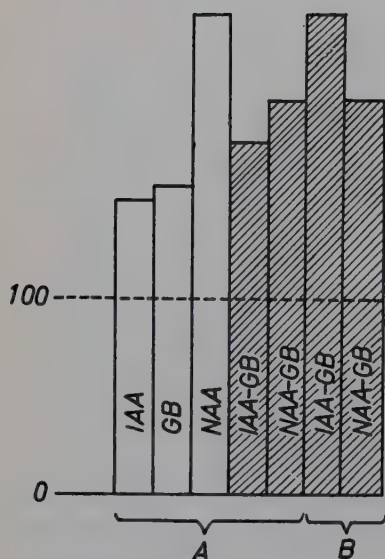


Figure 4. *Relative entrance water permeability*. A. after 3 hours. B. after 6 hours. Values as percentages of water controls.

Table 1. Entrance water permeability (Pw_{en}) of potato parenchyma under the action of 10^{-5} M GB, IAA, NAA and IAA-GB, NAA-GB respectively. Size of the cylinders 8 mm. ϕ \times 5 mm. $T=25.0^{\circ}\text{C}$. Mean values of 5 experiments. Values are calculated as percentages of Pw_{en} in dist. H_2O .

Substances	Exposure time	
	3 hrs.	6 hrs.
GB	164.7 ± 3.8	—
IAA	155.4 ± 6.2	—
NAA.....	252.9 ± 8.6	—
IAA-GB	184.0 ± 4.3	253.8 ± 11.9
NAA-GB	201.0 ± 5.0	208.1 ± 5.9

The details of the method and the calculations were already discussed in previous publications (Hasman 1954, 1957).

The comparative action of GB with IAA and NAA on the Pw_{en} , and its synergistic effect on the same process together with the latter compounds, is schematically represented in Figure 4, and tabulated in Table 1.

From the obtained results it is permissible to conclude that the effect of GB on the Pw_{en} process is almost equal to that produced by IAA, the increase in Q-value being 9.3; whereas the decrease in Q-value between GB and NAA amounts to 88.2. The above results clearly explain the trend of the water uptake-curves under the action of the applied compounds: the compound which enhances the entrance water permeability most also exerts maximum promotion in the water uptake.

After these findings, in order to verify whether the obtained interaction effect of GB with IAA and NAA is due to changes in the Pw_{en} , it was thought suitable to repeat the permeability determinations with the samples pre-treated in IAA-GB and NAA-GB respectively (cf. Figure 4).

From the schema it can be seen that at the 3rd hour the Pw_{en} under the action of IAA-GB is increased, this amounting to Q-value 28.6. On the other hand, the influence of GB on the permeability effect produced by NAA is diminished by Q-value 52.0.

Having observed the remarkably pronounced effect of GB on the water uptake process at the 3rd hour, and to be able to judge the validity of its synergistic effect on the permeability results of the same exposure time, it was thought necessary to perform similar experiments at the 6th hour.

The results of this set are shown in Figure 4 B.

As seen from the schema at the 6th hour, the effect of IAA-GB on the Pw_{en} reaches the highest point, thus producing a maximum result which equals in value that of NAA at the 3rd hour. This clearly explains the results obtained in the water uptake process, where the IAA-GB values are over and

above the already known drastic pure NAA effect. On the other hand, the 6th hour NAA-GB level remained almost stationary compared with the 3rd hour value, but fell considerably in relation with the pure NAA-level of the third hour exposure time.

In conclusion, it can be stated that in all the permeability determinations the results observed are in juxtaposition with the water uptake values obtained with the same compounds; namely that the compound or mixture which enhances the entrance water permeability most also exerts maximum promotion on the water uptake.

Discussion

The general reaction of gibberellin on higher plants has induced several investigators to analyse the different physiological functions of gibberellin (GB) and to compare them with those produced by synthetic and naturally occurring auxins. Among them, Brian *et al.* (1955 b) investigated various aspects of the GB-action of which the water uptake determinations for longer periods, using Hackett and Thimann's method on potato tissues, concerns especially this present study. These latter research-workers came to the conclusion that, although IAA and NAA increased the water uptake, GB had no detectable effect on this process. In this present research short period determinations were carried out using the technique of this laboratory. In general, the obtained results, for longer periods, are in accordance with those of Brian *et al.*, but it is worth noticing that the reaction of GB is almost parallel to that of IAA up to the 6th hour. This is due to the fact that, at earlier hours, GB increases the entrance water permeability almost as much as IAA does.

Ende and Koornneef (1960), sprayed the tomato stems with gibberellic acid solution and from the cryoscopic determinations of their experimental material, arrived at the conclusion that the compound gibberellic acid increased the osmotic pressure of the stem tissue cells. In the present work, the intersection point values obtained from the suction potential determinations, under the action of GB, showed no difference from those of IAA or NAA; thus denoting that there is no effect in the osmotic pressure under the influence of GB. This is in accordance with the previous investigations for other auxins, but is in contradiction with the results of Ende *et al.* According to their suggestion, gibberellic acid seems different in nature from other auxins, as auxins are not known in the literature to exert any osmotic changes.

The comparative effect of GB and IAA on the tensility of pea stems was studied by Yoda and Ashida (1958). These investigators observed that IAA

increases the tensility and plasticity, whereas gibberellin decreases both these responsible factors of the water exchange. Hence, according to Yoda *et al.*, GB has an opposite effect to that of auxins on the tensility process. From graphical analysis of S.P.-curves for longer periods (Brauner-Hasman 1949) and from the direct stretching measurements (Brauner-Hasman 1952), it was concluded that the tensility of the auxin treated material was enhanced at the longer periods (24 to 48 hrs.). This is in good agreement with the findings of Yoda *et al.* for the tensility effect of IAA.

According to the previous findings of Hasman (1943) the degree of deviation of the positive part of the S.P.-curve from the linear course, explains the limiting tensility of the cell wall. In this present paper the trend of the obtained S.P.-graphs of the GB treated material for the later periods, indicate the greatest divergence from the linear course in comparison with that of IAA and NAA, which show the least. This denotes that the GB treated material has a lesser tensility in contrary to the IAA and NAA treated material. This result is also in accordance with the findings of Yoda *et al.* and clarifies the decrease in the water uptake of the GB treated material for longer periods. This diminishing tensility of the cell wall seems to limit the obtained water uptake in the later hours.

Although various experiments are being performed by several workers on the interaction or synergism between auxins and gibberellin on the growth phenomena of different plants, the nature of the process is not yet clearly explained. This problem was studied in 1943 by Yabuta *et al.* on rice seedlings, but as uniform results were not obtained, a decisive conclusion could not be made. Later on, Nitsch and Nitsch (1956), using indole acetic acid-gibberellic acid, did not obtain an additive effect on the growth of oat coleoptiles, though in two cases an enhancement of synergism was observed. Brian and Hemming (1957), while analysing the interaction between gibberellic acid and IAA on the cell elongation of pea internode sections, observed that a significant synergism exists between gibberellic acid and IAA, but the influence of gibberellic acid depends on the presence of IAA. The synergistic effect of GB and IAA on shoot growth was studied in detail also by Kato (1958), and he arrived at the conclusion that, on the shoot growth process, GB acted additively or not additively depending on the concentration ranges of IAA and NAA. Kuse's experiments (1958) with sweet potato petioles indicate that in the absence of auxin the effect of GB is not promoting, but in the presence of the former it is stimulatory. According to Galston *et al.* (1959) when gibberellic acid is applied together with IAA to excised epicotyl sections of etiolated peas, their combined action on growth is not more but usually less than additive. On the other hand, if gibberellic acid is applied basally and IAA apically, the joint action of the compounds on growth is found to

be more than additive (synergistic). Galston and co-workers interpret the gibberellic acid-indole acetic acid interaction in terms of a "third factor" which provides an auxin-sparing mechanism.

Apart from the above mentioned auxin-gibberellin interaction on the growth phenomena, in the present research an analysis was made on the synergistic action of GB with IAA and NAA, upon the water uptake and its responsible factors. From the obtained results the following conclusions were drawn: in the absorption process the interaction between IAA and GB is much more pronounced than that between NAA and GB, except in the first three hours where the NAA-GB effect is slightly more noticeable; from the 6th hour onwards the IAA-GB curve rises even above the already known drastic graph produced by pure NAA. The cause of this observed augmentation at the early periods in the uptake process is due to the difference in the enhancement of Pw_{en} under the action of the applied compound. At the later hours, the action of IAA-GB shows a great effect on the tensility, this is proved by the observed small deviation of the S.P.-curves from the linear course. The comparable effect produced by NAA-GB on this process is reversed.

According to the results of this paper, in the earlier periods, gibberellin behaves like an auxin on both the water absorption process and one of its responsible factor, the Pw_{en} . In the later periods, GB acts in complete opposition, unlike an auxin, on the water exchange and its second component, the tensility. The third factor responsible for the water uptake, namely the osmotic pressure, remains unaffected under the influence of GB; in this case its action seems auxin-like.

The additive action of GB on IAA is promoting whilst its effect on NAA is retardative. Although many suggestions have been made to elucidate this phenomenon, the mechanism involved in it still remains obscure.

Summary

1. In the present paper, an attempt was made to compare the effect of GB alone and together with IAA and NAA upon the water exchange process, and its responsible factors, suction potential, tensility and water permeability. Potato tubers (*Solanum tuberosum*) were chosen as suitable experimental objects, and the determinations were carried out using the gravimetric immersion method.

2. GB had a noticeable auxin-like effect on the water uptake at the early period (3 hrs.) this being slightly above the value of IAA of the same exposure time. From the 6th hour onward, there appeared a gradual diminishment in the absorption process, the effect of GB at these later periods being dif-

ferent from that of an auxin. The obtained synergistic results of GB with IAA on the water uptake was remarkably high and significant throughout the whole experimental period compared to that of IAA; but GB with NAA brought forth a distinct retardation in relation to the pure NAA effect.

3. GB, in general, produced no effect on the S.P.-values, this being in accordance with the other auxins. The observed fall in the intersection point-values of the S.P.-graphs can be attributed to the degree of surplus water absorption, under the action of the applied compounds.

4. GB had a decreasing effect on the tensility of the cell wall, whereas the synergistic action of GB on IAA increased even more the known effect of IAA on the tensility.

5. The cause of the water uptake enhancement of GB at the 3rd hour was due to the increase in the Pw_{en} . During this period of time the synergistic action exerted by IAA-GB on the permeability process was less than that of NAA-GB. This value was reversed at the 6th hour in favour of IAA-GB whose action on the Pw_{en} by far exceeded that produced by NAA-GB.

In conclusion the authors wish to express their indebtedness to Prof. Dr. H. Tamiya and to the director of the Kyowa Fermentation Industry CO., Ltd., Dr. S. Kinoshita, for having kindly provided samples of gibberellin.

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Radioisotopic Study of Chlorophyll Accumulation in Soybean Leaves, in the Conditions of a Transfer from one Daylength to another ("Transfer-Effect")

By

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Introduction

Chlorophyll in the leaves is easily labeled through biosynthesis (Roux and Husson 1952, Shlyk and Godniev 1958, Brzeski and Rüker 1960); glycine- C^{14} and Δ -aminolevulinic acid- C^{14} are particularly efficient precursors.

It has been also shown that the accumulation chlorophyll does not follow exactly the same pattern when a given species is grown in short days instead of long days (Cheuvart 1954, Clauss and Rau 1956, Sironval 1957 a and b, Enloe 1959). This is particularly true for plants sensitive to photoperiodism. In this case, drastic changes in the pattern of accumulation of the green pigments can be obtained when the plant is transferred from one to another daylength at definite developmental stages. It is the "transfer-effect" which has been observed in *Fragaria vesca* (Sironval 1957 b) in *Cannabis sativa* (Cheuvart 1954) and in *Soja hispida* (Sironval 1957 a).

It appeared interesting to investigate this "transfer-effect" with isotopes and see what it could reveal of the biochemical relationship between the chlorophylls a and b.

Material and Methods

We use *Soja hispida*, variety Capitole. The plants are grown in two series — in short days (8 hrs) and long days (16 hrs) — at 20°C constant under artificial light of fluorescence Phytol lamps (about 5000 lux) from the seed to the appearance of

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the third leaf. At this time, one half of the plants grown in long days is transferred to short days, and one half of the plants grown in short days is transferred to long days. We obtain in this way four series:

1. Soybeans grown in long days, remaining in long days;
2. Soybeans grown in long days, transferred to short days;
3. Soybeans grown in short days, remaining in short days;
4. Soybeans grown in short days, transferred to long days.

Just at the moment of the transfer, all the leaves except the very young third leaf are removed. The removal of the leaves is done in such a way that the midrib and the petiole of the second leaf are left on the plant. This petiole is dipped during 24 hrs in a solution of Δ -aminolevulinic acid- 4-C^{14} (10 mg. or 200 μC /100 ml. solution).

The content of chlorophylls and the labeling of the two forms a and b is followed during the growth to the senescence of the third leaf (about 30 days) in the four sets of plants. The chlorophylls are extracted in acetone and the total quantity calculated following Mackinney (1941). An aliquot of the acetone extract is chromatographed on paper following Sironval (1954) and the chlorophylls a and b are eluted separately in ether. Chromatography according to Chiba and Noguchi (1954) has been performed several times in order to obtain a proper purification of the pigments. The quantities of the pigments in the eluates are measured following Comar (1942). The radioactivity is measured in a flow counter Tracerlab SC-16 working in the Geiger region.

Results

Figure 1 shows the accumulation of the total chlorophyll (a+b) per g. of fresh weight in the four series. It is seen that the transfer from long to short days depresses the accumulation in the early stage of growth of the leaf, whereas the transfer from short to long days accelerates the accumulation.

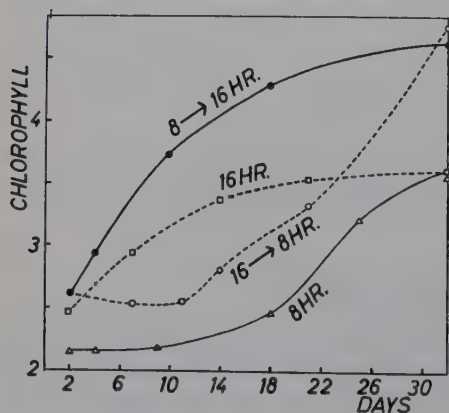


Figure 1. Course of the accumulation of total chlorophyll (a+b) in the four experimented series (expressed in mg. total chlorophyll per g. fresh weight of the third soybean leaf). Day-length given in the diagram.

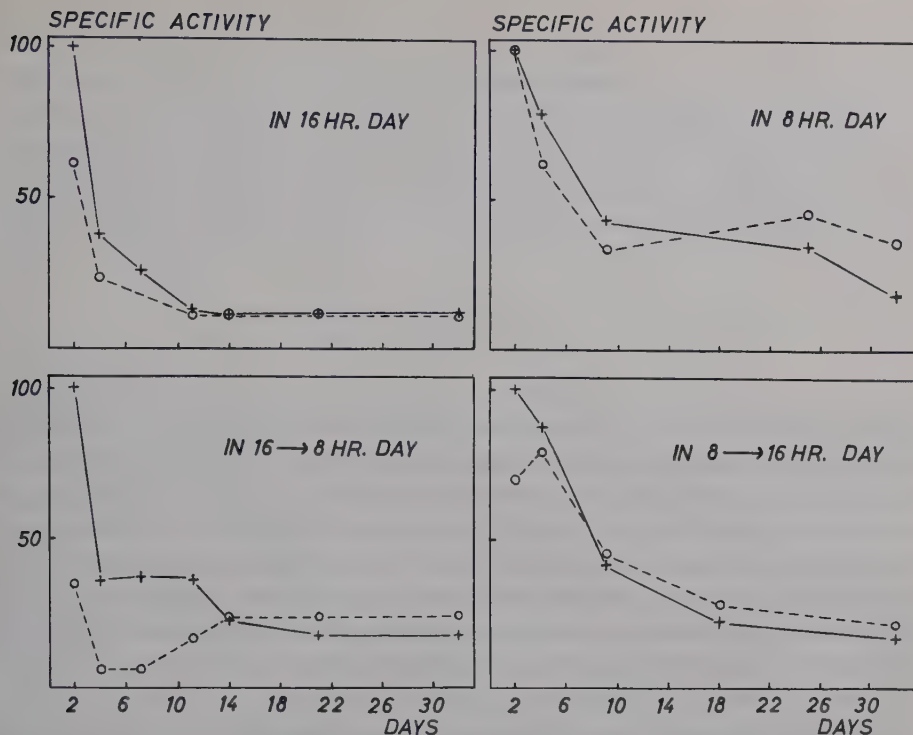


Figure 2. Evolution of the specific activity of the two chlorophylls a and b during the growth of the third soybean leaf in the four experimented series (expressed in relative units; 100=the specific activity of chlorophyll a the second day of the experiment).

The series transferred from short to long days accumulates much more chlorophyll than the series grown in long days permanently from the sowing. It is interesting to note that this "transfer effect" is obtained in very young leaves, in the absence of any adult leaves which have been removed just at the moment of the transfer. The very young leaves appear to be very sensitive to the transfer from one to another daylength.

Figure 2 gives the evolution of the labeling of the two chlorophylls a and b expressed as specific activities in relative units. A maximum of radioactivity is always reached two days after the beginning of the application of Δ -aminolevulinic acid; thereafter, the specific activity decreases rapidly. On the 10th to 15th day, it has reached a low level where it remains approximately constant until the end of the experiment, 10–15 days later.

The first 10–15 day period corresponds to the rapid growth and extension of the young leaf. During this period, the two pigments accumulate more

Table 1. Variations of the ratio $\frac{\text{specific radioactivity of chlorophyll a}}{\text{specific radioactivity of chlorophyll b}}$ in the four experimented daylength treatments.

Time in days	Series			
	16 hr-day	8 → 16 hr-day	8 hr-day	16 → 8 hr-day
2	2,0	1,4	1,0	2,8
4	1,6	1,1	1,2	6,2
6	1,4	0,9	1,3	8,0
20	1,0	0,8	0,9	0,8

or less rapidly in the leaf in the four experimental series. (Figure 1 expresses the quantity of pigments per g. fresh weight. When this quantity remains constant in the young leaf, this does not mean that there is no net pigment accumulation, since the fresh weight increases!) Therefore the observed decrease of the activity is certainly at least partially due to a dilution. It is difficult to evaluate the role played in the process by the pigment turnover.

The second 15—20 day period corresponds approximately to the expanded adult leaves. During this period the constancy of the label indicates a rather high stability of the two pigments in the chloroplasts.

During the first six days of the experiment, chlorophyll a is generally found more labeled than chlorophyll b. Table 1 gives the ratio $\frac{\text{specific radioactivity of chlorophyll a}}{\text{specific radioactivity of chlorophyll b}}$ $\left(\text{ratio } \frac{a^*}{b^*} \right)$ two, four, six, and twenty days after the application of Δ -aminolevulinic acid. It is seen that during the first six days of the experiment, the ratio $\frac{a^*}{b^*}$ reaches values equal to 1.0

or under 1.0 in two cases only. These lowest ratios are found in the series which have been grown under short days before the application of Δ -aminolevulinic acid. (8 hr-day and 8 hr → 16 hr-day series). The ratios of the series previously grown under long days are always higher (16 hr-day and 16 hr → 8 hr-day series). The highest ratios are found in the series which has first been grown under long days and has been transferred to short days (16 hr → 8 hr-day series). Here exceptional values up to 8.0 are recorded. This indicates that the photoperiodic treatment of the plants has an effect on the relative labeling of the two chlorophylls a and b, as it has on the accumulation of the pigments in the growing leaf.

Fifteen days after the beginning of the experiment, the labeling of chlorophyll b is equal or a little higher than that of chlorophyll a (see Figure 2 and table 1 after 20 days). The picture is very different from that of the first

six days. In particular the attentive consideration of the series transferred from 16 hr to 8 hr-day (Figure 2) shows that the evolution of the ratio from high values during the first days of the experiment to low values at the end of the experiment, occurs from the 7th to the 14th day by a simultaneous increase of the specific activity of chlorophyll b and decrease of the specific activity of chlorophyll a. This particular case may suggest a formation of some molecules of chlorophyll b from molecules of chlorophyll a.

Discussion

It is clear from the results that the accumulation of the two chlorophylls a and b in the soybean third leaf is influenced by the photoperiodic treatment given to the plants. This is not surprising since such effects are known for different plant species (Sironval 1957 a). We intend only to insist on the two following points:

1) The "transfer effect" is obtained in the young growing third leaf in the absence of any adult leaves. This means that the young leaf "remember" the conditions in which the previous adult leaves have been grown. When the plants are transferred from short to long days for instance, the young leaf "feels" the previous short day treatment: the transferred growing leaf accumulates much more pigments than the control leaf which has been permanently grown in long days. In this case, *it seems that something accumulates in short days which is able to increase the chlorophyll synthesis after the transfer to long days.*

2) The use of Δ -aminolevulinic acid labeled with C^{14} shows that, in the very young growing leaf, the relative labeling of the two chlorophylls a and b can vary according to the daylength treatment. In the young leaf the ratio $\frac{a^*}{b^*}$ is lower when the plants have first been grown in 8 hr-day than when the plants have first been grown in 16 hr-day. Some results seem to indicate a possible formation of some chlorophyll b molecules from chlorophyll a molecules. However, if it exists, such a process must be very slow: a rapid interconversion is excluded, since in our experiments the equalisation of the labeling of the two pigments requires several days; *it is thus very doubtful that the reversible transformation chlorophyll a \rightleftharpoons chlorophyll b — if it exists — plays any role in the dissociation of water molecules involved in photosynthesis* (see also Blass, Anderson and Calvin 1959).

A possible way to account for a slow transformation $a \rightarrow b$ consists in the assumption that the conversion of a molecule of a to a molecule of b could occur from time to time in the chloroplast, for instance as a result of an occa-

sional and local photooxidative process (depending on the local conditions of protection, light intensity, etc.). In fact, photooxidative experiments do not exclude the possibility that during photooxidation there is some production of chlorophyll b as a result of the oxidation of chlorophyll a (Sironval and Kandler 1958).

In our experiments photooxidation could be more or less easy according to the daylength treatment. For instance it is possible that the chloroplasts of the plants previously grown in long days have an internal organisation which protects the chlorophylls from photooxidation better than that of the chloroplasts previously grown in short days. In this case, photooxidation would be lower — and therefore the ratio $\frac{a^*}{b^*}$ higher — in the series first grown in long days, what we effectively find. A particular case would be that of the series grown in long days and transferred to short days. Here the internal protection to photooxidation would be good and the short duration of the light period after the transfer would not allow much photooxidation. In fact we find, in this case, exceptionally high $\frac{a^*}{b^*}$ ratios in full agreement with the suggested hypothesis.

Summary

The transfer of Soybean plants from one daylength to another greatly affects the accumulation of the chlorophylls in the leaves. This “transfer-effect” is studied using C^{14} Δ -aminolevulinic acid as tracer. It is shown that the relative labeling of the two forms of chlorophyll (a and b) varies according to the daylength treatment. A tentative interpretation of the facts is proposed. It is based on the assumption that in certain conditions, some molecules of chlorophyll b could be produced as a result of the photooxidation of some molecules of chlorophyll a.

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The Biosynthesis of Allantoin in *Symphytum*

By

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The ureides allantoin and allantoic acid occur widely in plants (Tracey 1955). Mothes and coworkers (1952, 1954, 1958) and Bollard (1957, 1959) have shown that in many plants these compounds, together with citrulline, assume a role in storage and translocation similar to that played by glutamine and asparagine.

Steward and Pollard (1957) have suggested that in plants allantoin and allantoic acid might be derived from glyoxylic acid, possibly by condensation with urea. There is some evidence for this pathway in basidiomycetes (Brunel and Brunel-Capelle 1951) and Frieberg, Bollard, and Hegarty (1957) reported that banana leaves fed urea- C^{14} incorporated radioactivity into allantoic acid. Krupka and Towers (1959) administered glycine- C^{14} and urea- C^{14} to whole wheat seedlings *via* the roots, and to wheat leaf disks by infiltration. Radioactivity was incorporated into allantoin and allantoic acid from glycine- C^{14} only, although the urea was readily metabolised. Also, only slight radioactivity could be detected in allantoin when glyoxylic acid- C^{14} was given.

The possibility that allantoin and allantoic acid may arise from purine degradation was demonstrated by Barnes (1959), who fed adenine-8- C^{14} to leaves of silver maple (*Acer saccharinum* L.) and found allantoic acid, urea and allantoin to be the most heavily labelled compounds in an ethanol extract.

In this paper, the question of allantoin biosynthesis has been further exam-

ined by administration of glycine-2-C¹⁴, hypoxanthine-8-C¹⁴, and urea-C¹⁴ to sterile root cultures and leaf disks of comfrey, *Symphytum uplandicum*, Nyman, (*S. peregrinum* of various authors). The use of sterile cultures of plant organs enables clear cut metabolic paths to be established. The successful culture of excised comfrey roots thus presented useful experimental material.

Experimental

C¹⁴-labelled compounds

Glycine-2-C¹⁴, hypoxanthine-8-C¹⁴, and urea-C¹⁴ were obtained from Amersham Radiochemical Centre, Harwell. Prior to use, they were examined for radiochemical purity by running paper chromatograms of aliquots and preparing radioautographs. The amounts added in the various experiments were: urea 15 μ C., 8.2 mC./mM; hypoxanthine, 10 μ C., 6.2 mC./mM; glycine, 10 μ C., 18.8 mC./mM.

Incorporation procedure

(a) *Leaf disks.* Disks 1 cm. in diameter were cut with a sharp cork-borer from selected young leaves and 2 g. fresh weight of disks was floated in an open petri dish on 20 ml. of water containing 10 or 15 μ C. C¹⁴-labelled chemicals, 2.5 mg. % penicillin and 6 mg. % neomycin. Absorption took place over 2 hours in sunlight at 25°; and leaf disks retained full turgor and normal appearance during the experiments.

(b) *Roots.* Excised comfrey roots were grown in the dark at 26°C for 5 weeks in 2.5 l. aliquots of a modified Whites medium (Boll and Street 1951). Iron was included at 1 ppm. as ferric ethylenediaminetetraacetic acid and the medium was supplemented with 250 ppm. Difco yeast extract. The pH of the medium after autoclaving was 5.4. Batches of approximately 2 g. fresh weight of root material were transferred to 500 ml. Kjeldahl flasks containing 20 ml. growth medium which included 10 or 15 μ C. of the C¹⁴-labelled chemicals. The flasks were gently shaken for periods of 2 or 8 hours in the dark at 26°C.

Extraction, chromatography, radioautography, and counting procedures

The tissue was quickly blotted dry and extracted for 5 min. with 100 ml. boiling 80 % (v./v.) aqueous ethanol, followed by two washings with 25 ml. 80 % ethanol (*cf.* Woodward and Rabideau 1953). The residue was extracted for 5 min. with 100 ml. boiling distilled water and washed twice with 25 ml. water. The aqueous extracts were freeze dried, and the ethanol extracts dried in a rotary film evaporator at 40°; known volumes of water were added and in the case of the ethanol extracts a small amount of water-insoluble material containing negligible radioactivity was centrifuged off and discarded.

Total radioactivity of the extracts was determined by drying small aliquots on to aluminium planchettes using an infra-red lamp and counting the radioactivity at infinite thinness using a Geiger-Müller tube (Phillips 18506) with a mica end-window (2 mg./cm.²).

Total radioactivity of the plant residues after alcohol and water extraction was determined by the combustion of 10–15 mg. aliquots of the dried, powdered material by the Van Slyke-Folch method, followed by counting of the C¹⁴ as barium carbonate (Sakami 1955). These results were corrected to the same geometry as the counts made at infinite thinness.

Radioautography

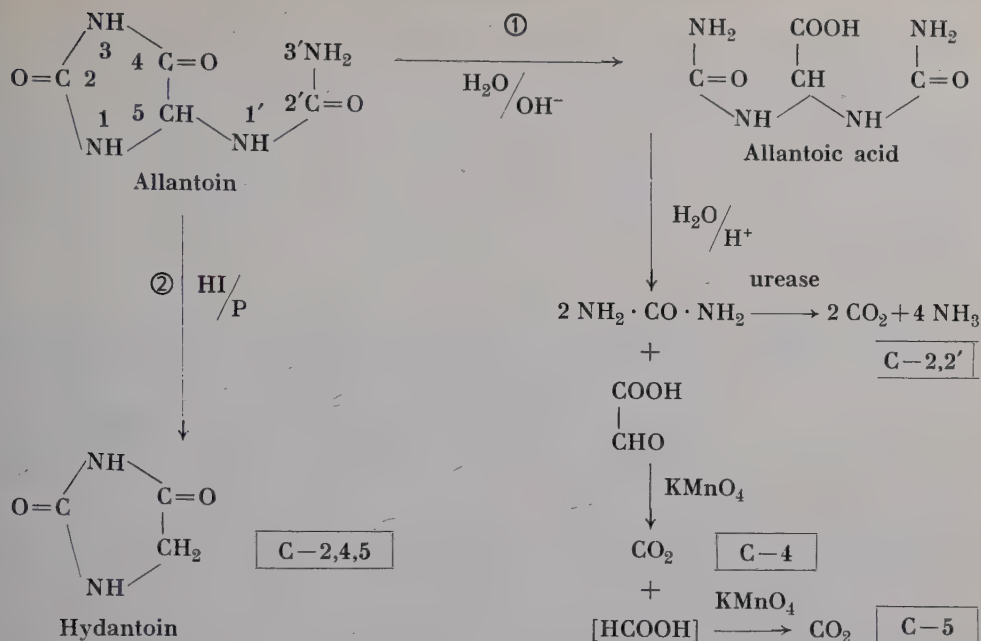
Aliquots of the extracts corresponding to 20–30 mg. fresh weight of tissue were taken for two-dimensional descending paper chromatography on oxalic acid-washed Whatman No. 1 papers in phenol saturated with water followed by butanol-acetic acid-water (25 : 6 : 25) (Bassham and Calvin 1957). Radioautographs were prepared using "Curix Rapid" Gevaert screen X-ray film with exposure times of two weeks. The radioactivity of the compounds detected was measured by counting directly off the paper using the Phillips 18506 GM tube. Large areas of radioactivity were subdivided into smaller areas which were counted separately. Where practicable all samples and spots were counted for a sufficient length of time to bring the standard deviation for the corrected count down to $\pm 5\%$.

Radioactive spots detected in this way were identified by superposition with spots detected with spray reagents for aminoacids (ninhydrin), carbohydrates (silver nitrate reagent), organic acids (aniline/xylose reagent), ureides (Ehrlich reagent, p-dimethyl-aminobenzaldehyde), (Lederer and Lederer 1957, Smith 1958). In several cases aliquots of the eluted radioactive substances were co-chromatographed with suitable quantities of suspected substances in a third solvent system. For carbohydrates, the solvent system ethyl acetate-water-pyridine (2 : 2 : 1) was used (Jermyn and Isherwood 1949). Paper ionophoresis was also employed, (in the apparatus of Markham and Smith, 1953), to separate organic acids in 0.75 *M* formic acid, pH 2.0, (Gross 1956) and ureides in borate-phosphate buffer, pH 8.0 (Zimmerman 1956).

Particular attention was given to establishment of the identity of labelled allantoin. The material was examined by two-dimensional chromatography, one-dimensional chromatography in *tert*-butanol-ammonia-water (100 : 5 : 20) and electrophoresis at pH 8 in borate-phosphate buffer (Zimmerman 1956). In all three cases the radioactivity coincided exactly with a spot detected by Ehrlich's reagent.

Degradation of allantoin

Labelled allantoin in ethanol extracts from comfrey roots fed hypoxanthine-8-C¹⁴ and glycine-2-C¹⁴ was separated by chromatography from other radioactive compounds present in the extracts on Whatman No. 3 filter-paper in phenol-water. The allantoin band was eluted and suitable amounts of carrier allantoin (50–400 mg.) were added to aliquots of the labelled material for carrying out the following two degradation procedures: —



(1) Allantoin was degraded to glyoxylic acid and urea by the method of Buchanan, Sonne and Delluva (1948). Glyoxylic acid was precipitated as the semicarbazone and combusted by the Van Slyke-Folch method to give the total radioactivity in carbon atoms 4 and 5 of allantoin. The individual radioactivities of carbon atoms 4 and 5 were determined by stepwise oxidation of glyoxylic acid semicarbazone with acid permanganate at 38° (Buchanan *et al.* 1948). Urea was degraded to CO_2 with urease (tablets, British Drug Houses, Ltd.) and the activity of carbons 2 and 2' thus determined.

(2) Hydantoin was obtained in 30 per cent yield from allantoin by reductive hydrolysis with phosphonium iodide reagent (Dalglish and Neuberger 1954, Blatt 1943). After recrystallisation from methanol, the product contained only traces of allantoin and ninhydrin-positive material (presumably glycine) and had M.Pt. and mixed M.Pt. $220\text{--}222^\circ$ (corrected). The hydantoin was combusted by the Van Slyke-Folch method to obtain the combined radioactivity of carbons 2, 4 and 5 of allantoin.

Results

Roots

Table 1 shows the relative amounts of radioactivity found in the roots and media for one experiment in which hypoxanthine-8- C^{14} , glycine-2- C^{14} , and

Table 1. *Percentage of radioactivity in media and comfrey roots after assimilation of glycine-2-C¹⁴, hypoxanthine-8-C¹⁴, and urea-C¹⁴ for 2 and 8 hours.*

Material	Urea		Glycine		Hypoxanthine	
	2 hr.	8 hr.	2 hr.	8 hr.	2 hr.	8 hr.
Medium ...	90.2	86.0	71.5	31.0	96.7	72.7
Roots.....	9.8	14.0	28.5	69.0	3.3	27.3

Table 2. *Percentage of radioactivity in the 80 % (v./v.) ethanol and water extracts and in the residual material of comfrey roots after assimilation of glycine-2-C¹⁴, hypoxanthine-8-C¹⁴ and urea-C¹⁴ for 2 and 8 hours.*

Material	Urea		Glycine		Hypoxanthine	
	2 hr.	8 hr.	2 hr.	8 hr.	2 hr.	8 hr.
Alcohol extract...	95.1	89.6	72.3	42.3	96.3	95.8
Water extract ...	1.4	3.1	3.4	1.5	0.9	1.9
Residue	8.5	7.2	24.2	56.3	2.6	2.5

Table 3. *Percentage radioactivity in compounds in the 80 % (v./v.) ethanol extracts after assimilation of glycine-2-C¹⁴ and hypoxanthine-8-C¹⁴ by comfrey roots for 2 and 8 hours.*

Compounds	Hypoxanthine		Glycine	
	2 hr.	8 hr.	2 hr.	8 hr.
Allantoin	79	97	4	13
Hypoxanthine	21	2	—	—
Serine	—	—	73	75
Glycine	—	—	20	6
Glutamine/citrulline...	—	—	—	0.5
Asparagine	—	—	—	trace
Origin	—	1	3	5

Radioactivities are recorded as percentages of the total amount of radioactivity detected after chromatography. Where a spot detected by radioautography had an activity of 5 counts per minute or less, the radioactivity is recorded as a "trace".

urea-C¹⁴ were fed for periods of 2 and 8 hours to comfrey roots. A greater proportion of glycine was absorbed than of either urea or hypoxanthine.

Of the C¹⁴ taken up, the relative amounts found in the various fractions are shown in Table 2. In all cases, a major portion of the absorbed radioactivity was found in the ethanol fraction and only a small proportion in the water extract. Where glycine was fed, substantial radioactivity was found in the "residue" fraction.

In Table 3 are shown the relative amounts of radioactivity found in various

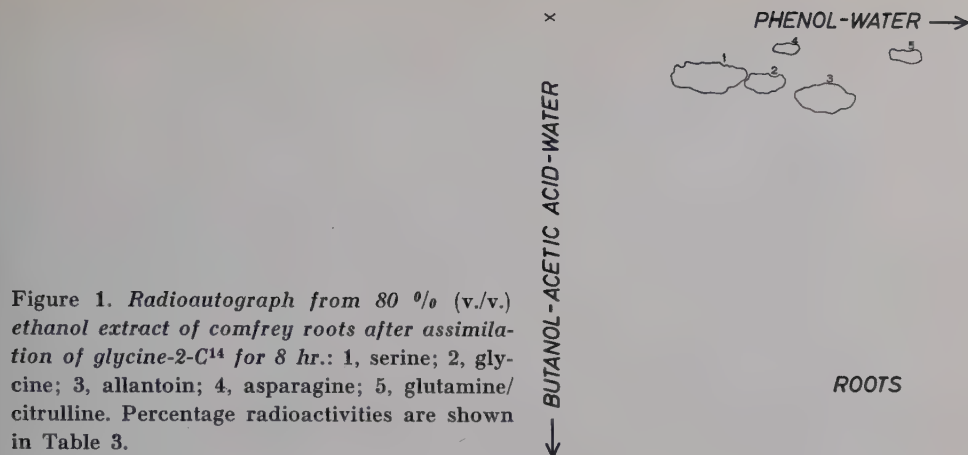


Figure 1. Radioautograph from 80 % (v.v.) ethanol extract of comfrey roots after assimilation of glycine-2-C¹⁴ for 8 hr.: 1, serine; 2, glycine; 3, allantoin; 4, asparagine; 5, glutamine/citrulline. Percentage radioactivities are shown in Table 3.

compounds separated by two-dimensional paper chromatography of aliquots of ethanol extracts from the roots fed glycine-2-C¹⁴ and hypoxanthine-8-C¹⁴. There was a marked formation of allantoin from both glycine and hypoxanthine and also for serine formation from glycine.

Radioautographs of ethanol extracts from roots given urea-C¹⁴ showed a number of faint spots with R_f's characteristic of amino- and organic acids, but no labelling of allantoin or allantoic acid was observed (cf. Figure 1).

Leaf disks

In Table 4 is shown the relative amounts of radioactivity in the ethanol extracts and residual material of leaf disks fed hypoxanthine-8-C¹⁴, glycine-2-C¹⁴, and urea-C¹⁴ over a period of 2 hours in one experiment. The activity in water extracts was negligible (approx. 1 %) and was not determined accurately. The amounts of radioactive material supplied and the specific activities were the same as in the experiment quoted with comfrey roots. As with the roots, a considerably higher proportion of the assimilated

Table 4. Percentage of radioactivity in 80 % (v.v.) ethanol extracts and residual material of comfrey leaf disks after assimilation of hypoxanthine-8-C¹⁴, glycine-2-C¹⁴ and urea-C¹⁴ for 2 hours.

Material	Urea	Glycine	Hypoxanthine
Alcohol Extract ...	89	52	88
Residue	11	48	12

Table 5. *Percentage radioactivity in compounds in 80 % (v./v.) ethanol extracts of comfrey leaf disks after assimilation of hypoxanthine-8-C¹⁴ and glycine-2-C¹⁴ for 2 hours.*

Compounds	Hypoxanthine-8-C ¹⁴ *	Glycine-2-C ¹⁴ **
Allantoin	71	trace?
Hypoxanthine	22	—
Sucrose	—	34
Aspartic acid	—	3
Glutamic acid	—	2
Alanine	—	4
Serine	—	8
Glycine	—	23
Asparagine.....	—	trace
Malic acid	—	11
Fumaric acid	—	4
Glyceric acid.....	—	4
Citric acid	—	trace
Succinic acid	—	trace
Origin	3	3

* 2 unknowns, thought to be nucleotides, contained 4 % of the total radioactivity.

** 11 unknowns, (Figure 2) contained traces of radioactivity.

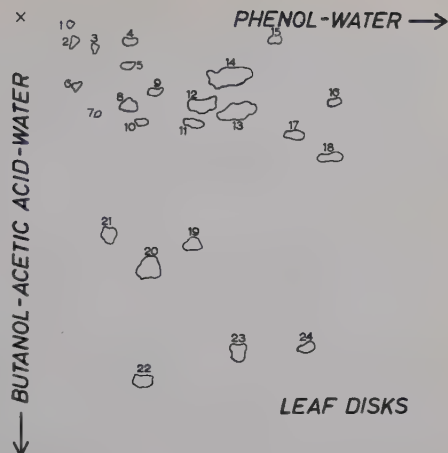
The radioactivities found in compounds on chromatograms are recorded as percentages of the total amount of radioactivity detected after chromatography. Where a spot detected by radioautography, had an activity of 5 counts per min. or less, the radioactivity is recorded as a "trace".

glycine remained in the "residue" fraction than was the case with hypoxanthine or urea.

The relative amounts of radioactivity found in various compounds after two-dimensional chromatography of ethanol extracts from leaf disks fed glycine-2-C¹⁴ and hypoxanthine-8-C¹⁴ are shown in Table 5. It will be seen that when hypoxanthine-8-C¹⁴ was fed, there was an extensive conversion to allantoin; two unknown spots were weakly labelled. Neither of these coincided with adenine, uric acid, guanine or xanthine, (*cf.* Barnes, 1960). Aliquots of the ethanol extract were chromatographed in butanol-acetic acid-water (80 : 20 : 20), dried and chromatographed in the same direction in acetone-butanol-water (80 : 10 : 10) (Dorough and Seaton 1954). In this way further confirmation of the identity of allantoin was obtained. The two unknowns were located in the nucleotide region of the chromatogram, but were not identified with certainty.

The feeding of glycine-2-C¹⁴ to leaf disks resulted in the labelling of a wide variety of compounds in the ethanol extract, with activity predominantly in sucrose, malic acid and serine, (Table 5, Figure 2). Small amounts of activity were identified in a number of amino-acids and organic acids. Spot No. 17 on the radioautographs (Figure 2) probably coincided with allantoin, but was only faintly labelled and could not be distinguished with certainty from

Figure 2. Radioautographs from 80 % (v./v.) ethanol extract of comfrey leaf disks after assimilation of glycine-2- C^{14} for 2 hr.: 8, aspartic acid; 11, glutamic acid; 12, serine; 13, glycine; 14, sucrose; 15, asparagine; 17, allantoin or threonine; 18, alanine; 19, glyceric acid; 20, malic acid; 21, citric acid; 22, fumaric acid; 23, succinic acid; 1—7, 9, 10, 16, and 24 unknowns. Percentage radioactivities are shown in Table 5.



threonine. Near the origin several unknowns were observed to be lightly labelled (Nos. 1—7, 9 and 10); these were located in the region where nucleoside di- and triphosphates and phosphorylated sugar derivatives are found (Bassham and Calvin 1957), but were not identified. None of these unknowns was allantoic acid, which is located near Nos. 5, and 9 (Figure 2).

The complete contrast in the metabolism of glycine-2- C^{14} by comfrey roots and leaf disks is emphasised by a comparison of the tracings of radioautographs in Figures 1 and 2. The radioactive areas have been outlined since the more faintly labelled spots could not be reproduced with clarity.

Radioautographs of ethanol extracts from leaf disks fed urea- C^{14} revealed a number of labelled compounds, including amino- and organic acids and sucrose. As in the case of roots, no labelling of allantoin or allantoic acid was observed so these results are not presented in detail.

Degradation of allantoin

Two samples of labelled allantoin were prepared from roots which had assimilated (a) hypoxanthine-8- C^{14} and (b) glycine-2- C^{14} .

(a) *Hypoxanthine-8- C^{14}* . (1) When labelled allantoin was degraded to glyoxylic acid and urea according to Buchanan *et al.* (1948), all the radioactivity was found to be located in the carbon atoms of urea (specific activity, 7,820 counts/min. \cdot mM carbon), with none detectable in glyoxylic acid. Hence the radioactivity in the allantoin was located in carbon atoms 2 and/or 2'.

(2) When labelled allantoin was degraded to hydantoin, the specific activities found were

Allantoin	5,540 counts/min. \cdot mM
Hydantoin	2,730 counts/min. \cdot mM

i.e. the recovered hydantoin had a specific activity which was 49 per cent of that in the allantoin. It can be concluded that carbon atoms 2 and 2' of allantoin were labelled in equal amounts.

(b) *Glycine-2-C¹⁴*. On degradation of allantoin by the procedure of Buchanan *et al.* (1948), the following distribution of radioactivity was found: —

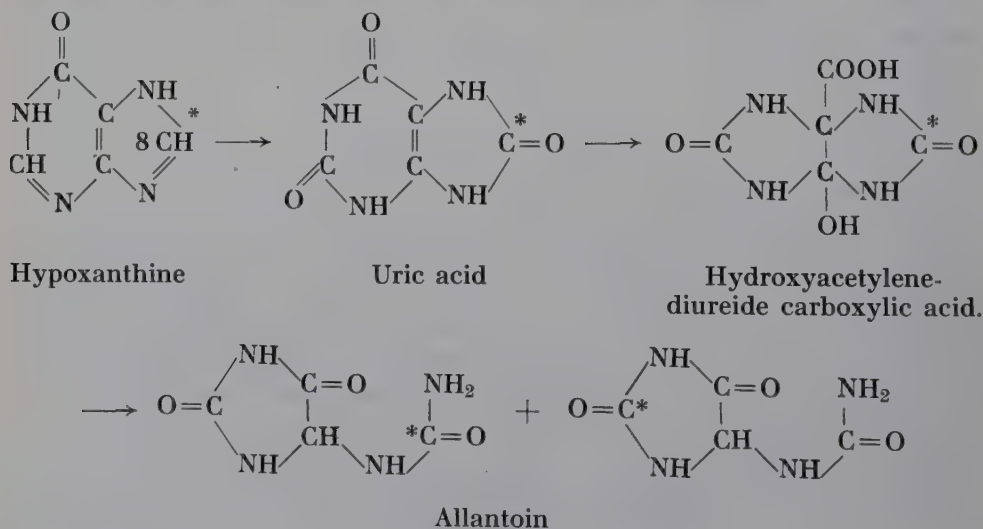
Carbon atom	Specific activity (counts/min. · mM carbon)	% Total counts
2+2'	1,920	27
5	1,062	16
4	3,844	57

Discussion

Krupka and Towers (1959) suggested that in plant tissues allantoin may be synthesised from glycine by way of purine precursors and that this process represents an important metabolic route for glycine in the roots of some plant species. Support for this thesis can be summarised as follows

(1) There was a marked conversion of hypoxanthine-8-C¹⁴ to allantoin in both roots and leaf disks of comfrey.

(2) Degradation of the allantoin-C¹⁴ extracted from roots fed hypoxanthine-8-C¹⁴ showed carbons 2 and 2' to be equally labelled. This would be expected only if allantoin-C¹⁴ were formed from uric acid-8-C¹⁴, *via* a symmetrical molecule such as hydroxyacetylene diureide carboxylic acid.



There is evidence that hydroxyacetylene diureide carboxylic acid is one of the unstable intermediate compounds formed by the action of mammalian uricase on uric acid (Canellakis and Cohen 1955, Hübscher, Baum, and Mahler 1957).

(3) Glycine-2- C^{14} was converted to allantoin in appreciable amounts by comfrey roots but not by leaf disks.

(4) Degradation of the allantoin- C^{14} extracted from roots fed glycine-2- C^{14} showed carbon atom 4 to be the most heavily labelled. Buchanan and his coworkers have shown by tracer and enzymic methods that in avian liver carbon atom 4 of the purine ring is derived from carbon atom 2 of glycine (Hartmann and Buchanan, 1959). They also showed that carbon atoms 2 and 2' of the purine ring are derived from formate. The presence of 27 per cent of the radioactivity in carbons 2 and 2' of allantoin- C^{14} from roots fed glycine-2- C^{14} is readily explicable if these atoms are derived from formate in plant tissues also, since the large conversion of glycine-2- C^{14} to labelled serine (Table 3) would be expected to result in a formate- C^{14} pool (Meister 1957). The differentiation of carbon atom 5 from carbon atom 4 in the degradation procedure used depends on the difference in two reaction rates and is therefore liable to error. The activity recorded in carbon atom 5 may have been partly derived from that in carbon atom 4.

(5) Under the conditions of these experiments, no radioactivity was observed in allantoinic acid, urea or glyoxylic acid when either hypoxanthine-8- C^{14} or glycine-2- C^{14} was metabolised by roots or leaf disks. When two-dimensional chromatograms were sprayed with Ehrlich's reagent appreciable quantities of urea were always detected, but allantoinic acid was either absent or present only in traces. Mothes and Engelbrecht (1954) examined fluctuations in the allantoin content of tissues of *Symphytum* species over the growing cycle and concluded that, particularly in root tissue, the formation of allantoin was primarily a mechanism for storage and translocation of nitrogen rather than a step in the process of purine catabolism. The results presented here are consistent with this conclusion. Further experiments are required to determine whether, under different physiological conditions, such as in older tissue, the catabolic sequence through allantoinic acid to urea becomes operative, as in silver maple leaves (Barnes 1959).

(6) In sharp contrast, feeding of urea- C^{14} did not result in the labelling of either allantoin or allantoinic acid.

The simple labelling pattern observed in radioautographs of ethanol extracts of roots metabolising glycine-2- C^{14} and hypoxanthine-8- C^{14} is not surprising, as the root tissues consisted almost entirely of mature, non-dividing cells. While there was a strong tendency for the conversion of hypoxanthine to allantoin in the leaf disks, the apparent lack of conversion of glycine to

hypoxanthine in the same tissue requires explanation and may have been due to a high requirement of glycine in other metabolic pathways.

The authors wish to thank Beris G. Butler for carrying out some preliminary experiments, Dr E. Wong for advice on the degradation procedures and W. D. Bennett and Margaret Hampton for skilled technical assistance.

Summary

Glycine-2-C¹⁴, hypoxanthine-8-C¹⁴, and urea-C¹⁴ were fed to both leaf disks and sterile root cultures of *Symphytum uplandicum* (Nyman). In both tissues, hypoxanthine-8-C¹⁴ was largely converted to allantoin, in which the distribution of radioactivity suggested its formation by way of a symmetrical intermediate. In leaf disks, the conversion of glycine-2-C¹⁴ to allantoin was at most very slight. In roots, it was partially converted to allantoin with a more complex pattern of labelling. Urea-C¹⁴ was not converted to allantoin in either tissue. It is concluded that in roots allantoin is synthesised from glycine through purine intermediates.

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Nature and Location of Phenolase in Germinating Lettuce

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In an earlier paper (Mayer 1954) the presence of polyphenol oxidase (equivalent to phenolase as defined by Mason 1957) in crude extract of lettuce seeds was shown. Recent work has indicated that it is incorrect to group all phenolases together as if only one distinct enzyme is being dealt with. Thus Clayton (1959) showed the presence of two distinct enzymes in tobacco leaves and Mayer and Friend (1960 a) were able to show that three distinct phenolases are present in sugar beet leaves, present respectively in the mitochondria, chloroplasts and the soluble part of the cell. A number of distinct phenolases have also been shown in leaves of *Solidago* (Björkman and Holmgren 1960). Moreover it was recently shown that a phenolase acting as DOPA oxidase could also oxidise DPNH (Mayer 1959). Because of these facts and because of the uncertainty regarding the terminal oxidase functioning in lettuce seeds it was decided to reinvestigate the phenolases in germinating lettuce seeds more thoroughly. Evidence has been obtained for the existence of two distinct phenolases, one soluble and one present in the mitochondria. The changes in the activities of these enzymes during germination will be described and also their response to germination stimulators and inhibitors.

Experimental

General Lettuce seeds of the light sensitive variety Grand Rapids were used throughout the experiments. The batch of seeds used gave 10 % germination in the dark. The seeds were germinated at 26° in the dark or in continuous light in petri dishes on filter paper in water or solutions to be tested.

Phenolase was assayed by following oxygen uptake by conventional Warburg technique. The reaction mixture contained usually 0.5 ml. substrate, 2×10^{-2} M, 0.5 ml. 0.1 M KH_2PO_4 , pH 6.3 and 0.5 ml. enzyme preparation in buffer (see below) and water to a final volume of 2.0 ml., with 0.2 ml. KOH (20 %) in the centre well. The latter was omitted when cyanide inhibition was studied. Experiments were carried out at 25° and results calculated for that period of the experiment during which oxygen uptake was linear with time. The results are expressed as $\mu\text{l O}_2/\text{minute}$ per 25 mg. original dry seeds or per mg. protein. Substrates were either of Analar quality or were purified by recrystallisation. The substrates used were 4-methyl catechol (Me-catechol) dihydroxyphenyl alanine (DOPA) caffeic acid, *p*-cresol, tyrosine, chlorogenic acid and quinol. In early experiments attempts were made to use catechol as substrate, but the reaction ceased after three minutes presumably due to reaction inactivation. These experiments were therefore discontinued.

Proteins were determined by the method of Lowry *et al.* 1951. All reagents were made up in distilled water which had been passed through an Amberlite Monobed MBI resin column.

Preparation of the enzyme. Seeds or seedlings were ground with 0.4 M sucrose, 0.1 M KH_2PO_4 , pH 6.3, (10–20 ml./g. dry wght.). The extracts so obtained was passed through cheese cloth, centrifuged at $1700 \times g.$ for 5 minutes and the residue discarded. The supernatant was centrifuged at $20000 \times g.$ for half an hour. The supernatant of this was used as the soluble enzyme, and the residue of the centrifugation was used as mitochondria. In earlier experiments the mitochondria were washed twice but later this washing was found to be unnecessary. The mitochondria were resuspended in the same sucrose buffer, using an all glass homogeniser for resuspension. Attempts at purification are described under results.

Results

As two distinct cell fractions were examined these will be described separately.

Soluble enzyme

The ability of the soluble enzyme, extracted after various periods of germination in the dark or continuous light, to oxidise a number of substrates was tested. The results are shown in Table 1. It can be seen that three of the substrates, DOPA, Me-catechol and caffeic acid are oxidised at about the same rate by the enzyme obtained in all the treatments. The phenolase activity does not appear to change either with time or with percentage germination. Seeds placed for 72 hrs in the light germinate to 100 % while those in the dark germinated not more than about 10 %. The ability of the soluble enzyme to oxidise *p*-cresol (*i.e.* cresolase activity), is very low and was characterised by a lag period of up to 2 hrs. The cresolase activity of the extracts was somewhat increased and its lag period decreased when the reaction was studied as a coupled oxidation of ascorbic acid in the presence

Table 1. *Phenolase activity of soluble extract of lettuce seeds germinated for various periods in dark or in continuous light. Substrate concentration 5×10^{-3} M. Rate of oxidation $\mu\text{l O}_2/25$ mg. seeds \cdot min.*

Time of germination hrs. and conditions	Quinol	DOPA	Me-Catechol	Caffeic Acid	p-cresol
0	13.7	15.5	17.5	15.4	0.47
24 Light	7.1	15.4	15.4	15.2	0.35
Dark	4.4	13.2	14.7	15.4	0.4
48 Light	10.5	13.5	15.5	17.3	0.14
Dark	10.2	14.5	15.0	16.2	0.21
72 Light	14.6	13.5	20.0	18.2	0.25
Dark	8.4	13.4	15.8	13.6	0.16

Table 2. *Ability of soluble phenolase to carry out oxidation of p-cresol in the presence of ascorbic acid. The extracts were exhaustively dialysed. Ascorbic acid 2.5×10^{-2} M p-cresol concn. 5×10^{-4} M. Rate of oxidation $\mu\text{l O}_2/25$ mg. seeds \cdot min.*

Treatment	Ascorbic acid	Ascorbic acid + p-cresol	Lag period
Dry seeds	0	2.1	10 min.
Germ. 24 hrs water	0	0.85	15 min.
Germ. 48 hrs water	0	0.82	15 min.
Germ. 48 hrs coumarin 5 mg. %	0	0.65	20 min.
Germ. 48 hrs thiourea 125 mg. %	0	0.81	20 min.

Table 3. *Phenolase activity of soluble extract of lettuce seeds germinated for 48 hrs. in water, thiourea 1250 ppm or coumarin 50 ppm. (Substrate conc. 5×10^{-3} M.) Rate of oxidation $\mu\text{l O}_2/25$ mg. seeds \cdot min.*

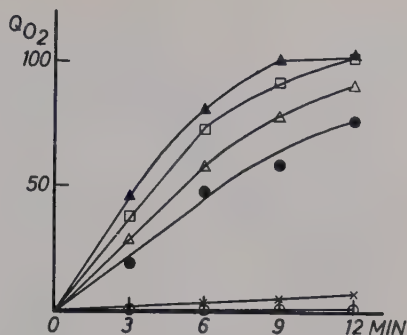
Treatment	Substrate				
	DOPA	Quinol	Me-catechol	Caffeic acid	p-cresol
Water	14.2	10.0	13.5	13.7	0.26
Thiourea	8.2	0	15.5	13.0	0.26
Coumarin	11.9	9.5	15.6	12.6	0.36

Table 4. *Effect of various inhibitors on phenolase activity of soluble extract of dry lettuce seeds. Results as % inhibition, control 0 %.*

Inhibitor concentration M		Substrates			
		Quinol	DOPA	Me-catechol	Caffeic acid
KCN	2.5×10^{-4}	100	92	88	92
Diethyldithiocarbamate	10^{-3}	100	95	51	88 % decreasing rapidly to 25 %
	5×10^{-4}	100	63		
	10^{-4}	100			
	5×10^{-5}	75			
	2.5×10^{-5}	44			17.5 % stimulation
Thiourea	10^{-3}	100	35	13	
	5×10^{-5}	60			

Figure 1. Oxidation of quinol by dialysed extracts of lettuce seeds in presence of boiled extracts or *Me*-catechol.

- (1) Original Extract ▲—▲
- (2) Original Extract dialysed ○—○
- (3) Dialysed extract+equivalent amount of boiled original extract ●—●
- (4) Dialysed extract+ 5×10^{-4} M *Me*-catechol □—□
- (5) As (3)+ 10^{-4} M thiourea ×—×
- (6) As (4)+ 10^{-4} M thiourea △—△



of *p*-cresol. Extracts of seeds given different treatments were therefore well dialysed so as to remove any endogenous substrate capable of oxidising ascorbic acid by a such coupled oxidation. Ascorbic acid and *p*-cresol were then added. The results shown in Table 2. It will be seen that cresolase activity is still very low and is not altered by the germination of the seeds in coumarin or thiourea. As before (Table 1) there was a drop in cresolase activity during the first twenty four hours of germination. The effect of the germination inhibitors and stimulators was also studied on the other substrates of phenolase and on the oxidation of *p*-cresol in the absence of ascorbic acid. The results are shown in Table 3. The oxidation of DOPA is somewhat depressed confirming earlier results (Mayer 1959), while the oxidation of *Me*-catechol, caffeic acid or *p*-cresol was hardly affected. However the fact that oxidation of quinol is completely depressed by germination in thiourea is very remarkable.

The effect of a number of inhibitors *in vitro* on the oxidation process in the presence of the substrates was studied, the results being shown in Table 4. These results point to a very marked difference in response between quinol and all the other substrates examined, thiourea again very markedly depressing oxidation of quinol but not of the other substrates.

This suggested that the oxidation of quinol is effected in some way which was quite different from that of the other oxidations. This view is strengthened by the results of experiments using dialysed extracts. Extracts were therefore exhaustively dialysed and tested against the various substrates. It was found that quinol was no longer oxidised by such dialysed extracts while the other substrates were oxidised at substantially their original rate. Addition to such a dialysed extracts of an equivalent amount of boiled extract restored the oxidation of quinol to about 60 % of its former rate. The same or even greater restoration of activity was obtained by the addition of 5×10^{-4} M *Me*-catechol, caffeic acid or chlorogenic acid (Figures 1, 2).

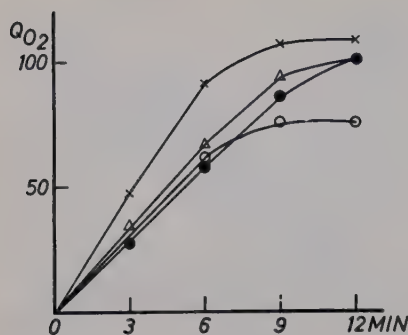


Figure 2. Oxidation of quinol by dialysed extract of lettuce seeds in presence of caffeic acid or chlorogenic acid.

- (1) Dialysed extract + 5×10^{-4} M caffeic acid \times — \times
 (2) Dialysed extract + 5×10^{-4} M chlorogenic acid \circ — \circ
 (3) As (1) + 10^{-4} M thiourea \triangle — \triangle
 (4) As (2) + 10^{-4} M thiourea \bullet — \bullet

Thus it is quite clear that the oxidation of quinol is by a coupled oxidation with a second phenolic compound which is mediated by phenolase. One important difference between restoration of activity by boiled extract and by added phenolic compounds must be noted. In the case of adding boiled extracts complete inhibition was obtained in the presence of 10^{-4} M thiourea. Whereas in the case of the added phenolic compounds the inhibition caused by this concentration of thiourea amounted only to 10—30 %. The endogenous phenol acting as coupling agent is apparently different from those used here.

An attempt to fractionate the enzyme was next made. The soluble enzyme (100 ml.) equivalent to 10 g. dry seeds was diluted with an equal volume of water and, to 200 ml. of this extract 10 ml. of calcium phosphate gel were added. After standing for 10 minutes the suspension was centrifuged and the supernatant discarded. The residual gel was eluted with 1.0 M phosphate buffer pH 6.3. The eluate was dialysed and reabsorbed on fresh phosphate gel. The gel was fractionally eluted with 0.1 M, 0.2 M and 0.3 M phosphate buffer pH 6.3. Most of the activity resided in the fraction eluted with 0.2 M buffer.

The eluate obtained at 0.2 M was dialysed and then made 0.3 M with respect to KCl and 0.2 M with respect to phosphate buffer. At this molar concentration the enzyme is not adsorbed on diethylaminoethyl-cellulose columns. It was then passed through a diethylaminoethyl cellulose column charged with phosphate buffer pH 7.7. The eluate was dialysed exhaustively. A precipitate formed which was separated from the solution by centrifugation. The residue was resuspended in phosphate buffer and its activity measured.

The activity of the original extract and the purified enzyme are shown in Table 5. No indication of separation of activity towards the three substrates is seen. The soluble enzyme was tested for activity against combinations of substrates. When the soluble enzyme was tested against each of the three substrates at a concentration of 5×10^{-3} M the oxygen uptake per 25 mg. seeds/min. was 6.7 μ l for DOPA, 7.8 μ l for Me-catechol and 7.1 μ l for caffeic acid. When the substrates were used in combination, each again being

Table 5. *Effect of purification of soluble enzyme on activity towards different substrates.*
Rate of oxidation $\mu\text{l O}_2/\text{mg. protein} \cdot \text{min.}$

Preparation	DOPA	Me-catechol	Caffeic acid
Original Extract	3.0	2.8	2.7
Eluate from Ca phosphate gel. (1.0 M phosphate buffer)	6.2	7.9	9.3
Eluate from second absorption on Caphosphate (0.2 M phosphate buffer)	13.6	12.5	16.4
Purified enzyme	42	45	47

present at a concentration of $5 \times 10^{-3} M$, the rates of oxidation were DOPA + Me-catechol 8.5 μl , DOPA and caffeic acid 7.4 μl and Me-catechol and caffeic acid 6.8 μl . If several enzymes were present it would be expected that each substrate is oxidised independently of the other and that the total oxygen uptake as a result would be additive. These results therefore indicate that only one enzyme is involved in the oxidation of the three substrates by the soluble enzyme.

Mitochondrial enzyme

The ability of the enzyme present in the mitochondrial fraction to oxidise the various substrates is shown in Table 6. It will be seen that mitochondria appear to lack entirely cresolase activity as well as the ability to oxidise quinol. The latter may be absent because the particles do not contain the natural carrier. In order to determine whether a genuine mitochondrial enzyme was involved or perhaps a lysosome fraction, the mitochondria were fractionated by differential centrifugation. It will be seen that the highest specific activity resides in the fraction precipitating between 12000 and 20000 $\times g$. (Table 7).

All the fractions including the fraction 12000—20000 g . have been shown to contain cytochrome oxidase activity (Poljakoff-Mayber and Mayer un-

Table 6. *Phenolase activity of mitochondria isolated from lettuce seeds germinated 48 hrs. in the light* (Substrate concn. $5 \times 10^{-3} M$ Mitochondria isolated at 20000 g .).

Substrate	Rate of oxidation $\mu\text{l O}_2/\text{mg. protein} \cdot \text{min.}$
Me-catechol	13.2
Dopa	4.0
Caffeic acid	13.2
Tyrosine	0
Quinol	0
<i>p</i> -Cresol	0

Table 7. *Phenolase activity of mitochondria isolated from 48 hrs. germinated lettuce seed and precipitated at different values of g. Substrate concentration 5×10^{-3} M. Rate of oxidation $\mu\text{l O}_2/\text{mg. protein} \cdot \text{min.}$*

Fraction ppt.	DOPA	Me-catechol
1700—6000 g.	2.5	5.0
6000—1200 g.	6.5	11.3
12000—20000 g.	9.2	20.0

published) and may therefore be regarded as genuine mitochondrial fractions. A striking feature of the data in Tables 6 and 7 is the difference of capacity of the mitochondria to oxidised DOPA and Me-catechol. This difference existed also if the seeds were germinated for 40 hours in thiourea (Table 8). The specific activity after treatment with thiourea is somewhat higher than in these prepared from seeds germinated in water in the dark, but lower than those of seeds germinated in the light (cf. Tables 6, 8). This is probably due to differences in contaminating proteins present in the fraction precipitating between 17000 and 20000 g. The enzyme present in the mitochondria also differed from the soluble one in being totally inhibited by 10^{-3} M diethyldithiocarbamate thus showing greater sensitivity to this inhibitor. An attempt to investigate the basis for the difference in rate of oxidation of DOPA and Me-catechol was made. As this might be a permeability effect, mitochondria were treated with 1 % desoxycholate in phosphate buffer pH 6.3 and their oxidation of the substrates then tested. No marked difference resulted from such treatment. The rate of oxidation was $5.6 \mu\text{l/min.} \cdot \text{mg. protein}$ with DOPA as substrate and $14.0 \mu\text{l}$ with Me-catechol in the untreated mitochondria and $6.5 \mu\text{l}$ and $14.0 \mu\text{l}$ with the same substrates in desoxycholate treated ones. This type of treatment had been found very effective in increasing activity in chloroplasts (Mayer and Friend 1960 b). Thus permeability seemed not to be the basis of the difference observed.

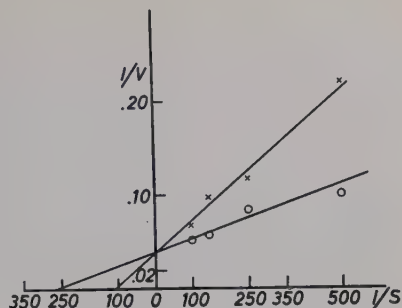
When the apparent Michaelis constant of the mitochondria towards DOPA and Me-catechol was determined, some difference was noted (fig. 3). The K_m for DOPA being about 10^{-2} M while that for Me-catechol was 4×10^{-3} M.

Table 8. *Phenolase activity of mitochondria isolated at 20000 g. from seeds germinated 48 hours in water or in thiourea 1250 ppm, in the dark. (Substrate concn. 5×10^{-3} M). Rate of oxidation $\mu\text{l O}_2/\text{mg. protein} \cdot \text{min.}$*

Treatment	DOPA	Me-catechol
water	4.04	7.0
thiourea	6.2	10.2

Figure 3. Relation between substrate concentrated ($1/S$) and rate of reaction $1/V$ for oxidation of Dopa and Me-catechol by mitochondrial fraction prepared from lettuce seeds germinated for 48 hours.

X Dopa
O Me-catechol



When additivity of oxidation was tested at substrate concentrations corresponding to the K_m values, no indication of such additivity was found (see also above).

It may be concluded therefore that the mitochondria contain one enzyme with different affinities towards the two substrates.

Discussion

It is quite clear from the foregoing that two distinct enzymes are present in lettuce seeds. One is located in the mitochondria and shows only catecholase activity while the other is a soluble enzyme and shows both catecholase activity and a rather low but clear cresolase activity. The enzymes differ not only in their location and relative cresolase activities but also in their affinity towards dihydroxy phenols and in their sensitivity towards inhibitors. Lettuce seeds do not contain a laccase but can oxidise *p*-dihydric phenols by a coupled oxidation. They can also, in a similar fashion oxidise ascorbic acid (Stavy and Mayer unpublished). Evidence for such coupled oxidation brought about by phenolase is lately accumulating, *e.g.* Neuman *et al.* 1958 Tomaszewski 1959, Mayer and Friend 1960 a. What is as yet unclear is the possible function of these phenolases. Butler (1960) has shown that lettuce seeds contain large amounts of chlorogenic acid which is not oxidised during germination. On the other hand it has frequently been observed in our laboratory that extracts of lettuce seeds germinated in thiourea fail to darken on standing while those from seeds germinated in water darken very rapidly. Despite this thiourea did not depress the oxidation of either caffeic acid or Me-catechol and that of DOPA was only slightly depressed. Only the oxidation of quinol and ascorbic acid, by coupled reactions, was completely inhibited by thiourea *in vitro* and *in vivo*. The oxidation of other substrates is only effected *in vitro* by much higher thiourea concentration.

It appears that the darkening reaction is concerned with the oxidation of some endogenous phenol, which is probably not chlorogenic acid, by a coupled oxidation. During germination the other phenolic compounds present in the seeds also change very little (Ashbel and Mayer unpublished).

A further important feature of the phenolase in lettuce seeds is that the soluble one does not increase in activity for the first 72 hrs of germination. This is not fully in agreement with earlier results on a different variety of lettuce seeds (Mayer 1954) when crude extracts were used but fully confirms results obtained earlier on the same variety Grand Rapid (Mayer 1959). The lack of increase in phenolase activity during germination on the one hand and the relative stability of all the phenolic compounds in the seeds make it difficult to assign a function to the phenolase. The low cresolase activity of the soluble enzyme and its apparent absence in the mitochondria indicate that this activity is probably of little importance. All attempts to show that the mitochondrial enzyme functions as a terminal oxidase have so far failed, very probably because of unsuitable experimental conditions. However further attempts to establish or refute this function of the phenolases are being made. It seems possible that it may function in this way during the very early stages of respiration and is then rapidly replaced by cytochrome oxidase. A further step in this investigation will be to determine how the two phenolases distribute between different organs of the plant when the seedlings are somewhat older and at what stage, if at all, phenolase appears in the chloroplasts (Mayer and Friend 1960 a, b).

Summary

A soluble and a mitochondrial phenolase are shown to occur in germinating lettuce seed. The activity of these enzymes towards five different phenolic substrates at different stages of germination was investigated. Evidence for the ability of the soluble phenolase to oxidise quinol by a coupled oxidation is brought. The effect of a number of inhibitors on the enzymes is described. The soluble enzyme was partially purified. Possible functions of the phenolases are discussed.

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Dwarfism of Peas and the Action of Gibberellic acid

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I. Introduction

In a series of experiments on the influence of gibberellic acid (GA) on the growth of several dwarf peas, it was stated that isolated internode sections and intact plants responded differently to GA. If the compound was applied to intact plants under greenhouse conditions, the dwarfs grew all to the same length and reached the size of a tall variety. This implies that the smallest dwarf shows the strongest response. When contrarily isolated sections of the same varieties were incubated in a GA solution, the sections of the tall variety grew fastest and of the dwarfs slower. In the present investigation an attempt was made to explain this divergence. First the growth of various dwarfs was analyzed and secondly the influence of GA on the growth of 1. intact plants, 2. isolated internodes.

II. Literature

For literature about GA we may refer to the many reviews *e.g.* by Stowe and Yamaki (1957) and Brian (1959) from which it can be read that one of the most characteristic phenomena caused by GA is the elongation of stem internodes of dwarf plants. This characteristic seems so specific that it justifies the definition of the gibberellins.

Furthermore it can be concluded from the literature that the gibberellins are generally considered as



Figure 1. The four varieties grown under greenhouse conditions.

- a. substances which, native in the plant, are present in a limited quantity in those cases in which an application produces an effect.
- b. interfere with a reaction, in which most likely light or auxin take part.

III. Material and Methods

The material was a "normal" pea and three dwarfs. The normal one was *Alaska*; the dwarfs were *Meteor*, *Gloire de Quimper* and *Petit Breton*. About the origin of the dwarfs it is known that

Meteor originated from *Pilot* × *Gem* and was selected by the firm Charles Sharpe at Sleanford (England)

Gloire de Quimper from *Express Alaska* × *Chemin Long* by Ets. Tézier at Valence (Drôme) (France)

Petit Breton from *Express Alaska* × *Nain très hâtif d'Annonay* by Ets. Simon Louis frs. Bruyères-le-Chatel (France)

It can be assumed that the dwarfs are mutants, though nothing is known about their genetics.

Cultivated under greenhouse conditions, the peas show the habits of Figure 1.

Methods. The growth of intact plants was determined both under greenhouse conditions and in conditioned rooms with artificial illumination and constant temperature (20°C). Light intensities were 33,000, 12,000, 3300, 1200, 350, and 100 ergs/sec and cm² cross section sphere (spherical photometer of Wassink and van der Scheer (1951). The lamps were fluorescent tubes (Philips-T.L.29) or Na-lamps.

Isolated internodes were cut from plants cultivated under artificial illumination (24 fluorescent tubes of 20W Philips T.L.29-intensity 2000 ergs/sec · cm² cross section sphere under a constant temperature of 20°C. During the test period of growth the same conditions prevailed.

In all experiments the mean error was calculated according to the formula

$$f = \sqrt{\frac{\sum \alpha^2}{n(n-1)}}$$

IV. Growth of intact plants under various conditions

A. Growth in darkness

When grown in darkness (air-conditioned room of constant temperature 20°C and relative humidity 50 %) the three dwarfs reached the same size as the "normal" variety (Alaska) which can be seen from Table 1. This simple fact reveals that the dwarfs are not always dwarfed, but that their dwarfism is determined by light. In other words, *in the dwarfs growth is reduced by light*.

Table 1. Length of peas grown in darkness, 20°C. 11 days after sowing; mean of 25—30 plants.

Alaska	18.7 ± 0.6 cm.
Meteor	20.3 ± 0.9 "
Gloire de Quimper	19.5 ± 0.6 "
Petit Breton	19.0 cm.

B. Growth under various light intensities

All four varieties were grown in continuous light of the five different intensities and in darkness.

The results of the growth measurements are shown in figure 2. Figure 2 shows that under the highest light intensity (I) all four varieties are small but nearly of the same size. In darkness they are tall but also of the same size. In between are the other intensities; the difference between *Alaska* and the dwarfs is greater as the light intensity is lower.

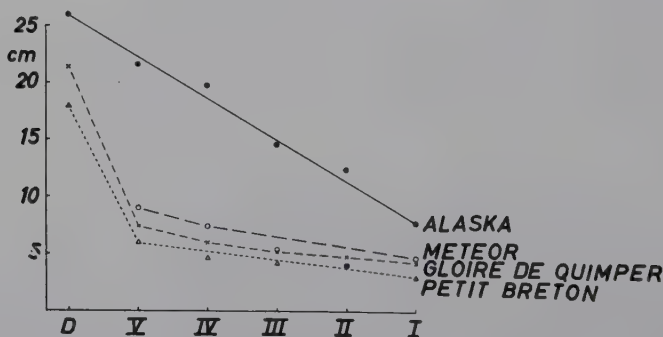


Figure 2. Length of the four varieties of dwarf peas, grown under five different intensities of white light and in darkness.

D=darkness	III= 3300
V= 350 ergs/sec · cm ² cross section sphere	II=12,000
IV=1200	I=33,000

It can be seen that in *Alaska* the reduction of growth is directly proportional to the intensity. In the dwarfs, however, the lowest intensity (V) reduces growth already as much as the highest (I) in *Alaska*. From this it can be concluded that the dwarfs are roughly 100 times more sensitive to the light than the "normal" pea.

C. Growth in various intensities of yellow light

In connection with the more or less unexpected fact that growth of the dwarf peas is reduced by light, light of narrow spectral ranges was used *viz.* yellow, red and blue. Not all data will be presented here, because the effects turned out to be essentially, though not quantitatively, the same, *viz.* always a reduction of growth occurred. Only the data of the yellow light are presented. The four varieties were cultivated in continuous light of NA lamps of the same intensities as the white *viz.* 33,000, 12,000, 3300, 1200, 350, 120, and 40 ergs/sec · cm² cross section sphere. Growth was measured after 11 days. The results are shown in figure 3. Again, in darkness all four varieties are of the same size (within the limits of the experimental errors). At the various intensities reduction of growth of the dwarfs is apparent; the higher the intensity, the more growth is reduced.

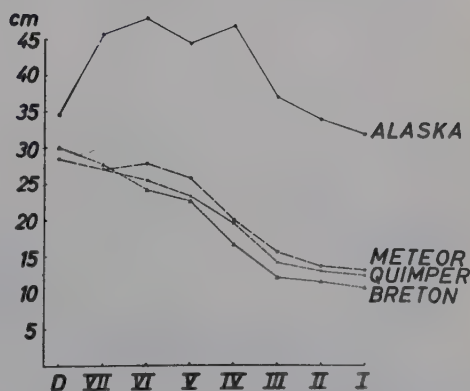
A comparison of the figures 2 and 3 makes it evident that the effect of yellow light is another than that of white light.

Alaska shows no growth reduction, except a very slight one at the highest intensity, but at the lowest intensities it shows the phenomenon of "yellow-etioliation", *viz.* more growth at low intensities than in darkness.

The growth reduction of the dwarfs is much smaller than at the same intensities of white light.

Figure 3. Length of the four varieties grown under seven intensities of yellow light.

D = darkness
 VII = 40 ergs/sec · cm² cross section sphere
 VI = 130
 V = 400
 IV = 1200
 III = 3600
 II = 11,000
 I = 33,000



The effect of light of narrow spectral ranges on dwarfism is an interesting problem indeed and investigations will be continued along this line. However the main purpose of this study was the interrelation between GA and dwarfism.

The conclusion of the growth measurements is that light is the factor that causes dwarfism in the dwarf peas.

D. Influence of GA on growth in various light intensities

The four varieties were grown at the same light intensities and supplied with a lanolin paste of GA (Gibberellic acid, I.C.I.) in a total quantity of respectively 66 μg per plant, 660 μg per plant, and 6600 μg per plant (on three successive days a quantity of 22 μg per plant of concentrations 1, 10, and 100 mg/l was applied). The results of one of these experiments are shown in figure 4. In this experiment a total quantity of 660 μg GA per plant was applied as a lanolin application to the epicotyl of the young seedling (6 days after sowing) which was still curved at that moment. Growth was measured 12 days after sowing. Figure 4 shows that:

- a. Dark grown plants respond slightly to GA.
- b. In Alaska there is *no* relation between light and GA. The difference in length between plants with and without GA is at all intensities approximately the same.
- c. The dwarfs contrarily respond to this quantity of GA. At the low light intensities the growth reducing effect of light is so small that the GA effect dominates, whereas at the high intensities the reduction of growth by light is reduced by the GA effect.

From the joint data it is possible to make a rough estimation of the GA quantity that balances the reduction of a special light intensity. Then we find:

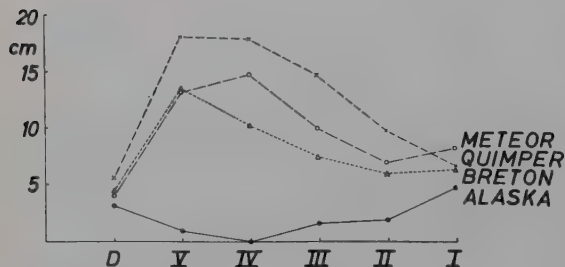


Figure 4. Difference in length between plants with and without GA in darkness and in five intensities of white light. The light intensities are the same as in Figure 2.

66 $\mu\text{g/plant}$: *Alaska*- growth reduction of all intensities remains.

Dwarfs- growth reduction of intensities $< 12,000\text{—}3300$ ergs/sec $\cdot \text{cm}^2$ cross section sphere remains.

660 $\mu\text{g/plant}$: *Alaska*- growth reduction of all intensities remains.

Dwarfs- all growth reductions are eliminated.

6600 $\mu\text{g/plant}$: *Alaska*- growth reduction of all intensities remains.

Dwarfs- all growth reductions are eliminated.

d. The sensitivity of the various dwarfs to GA differs.

Gloire de Quimper is the most sensitive variety with an optimum at 660 $\mu\text{g/plant}$;

Petit Breton and *Meteor* have their optimum at 6600 $\mu\text{g/plant}$ or higher.

V. Isolated sections

Sections of 10 mm. of the third internode of light grown plants were used. The plants were grown in continuous light of intensity 1980 ergs/sec $\cdot \text{cm}^2$ cross section sphere and constant temperature of 20°C . When the third internode was ± 12 mm. long (which under these experimental conditions lasts 14 days), 10 mm. sections of this internode were cut and incubated in the test solution. Ten sections in 10 ml. solution in 9 cm. Petri dishes were used. These were put in the same room under the same light conditions.

In preliminary experiments it was found that in darkness the effect of GA on growth is nearly zero; therefore all growth tests took place in light.

In other preliminary experiments the influence of P_H of the solution was investigated. Figure 5 presents the results and shows that the growth is less

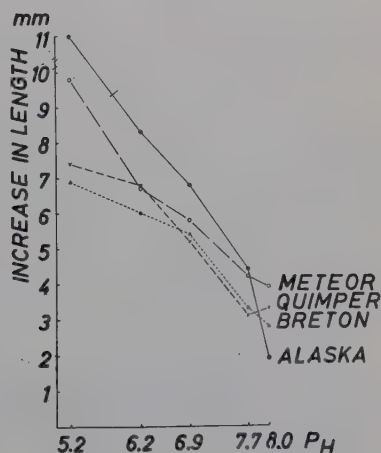


Figure 5. The relation between the P_H of the test solution and the growth of isolated sections.

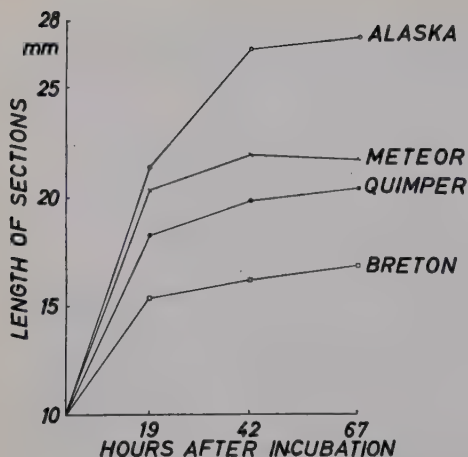


Figure 6. Length of isolated sections of the four varieties in sucrose+IAA+GA.

as the P_H values are higher. Therefore in all experiments the solutions were buffered at P_H 5.2 with a K-phosphate-buffer.

In preliminary experiments it was also stated that for GA to be effective, it needs the presence of sucrose and IAA. The optimal concentrations were 2 % sucrose and a ratio of IAA to GA of 100 to 1. Therefore in all tests 2 % sucrose was added to the solution and nearly always 10 p.p.m. IAA to 0.1 p.p.m. GA.

Growth with GA, IAA and sucrose

With sucrose 2 %, IAA 10 p.p.m. and GA 0.1 p.p.m. the four varieties respond as in figure 6 *viz.* sections of the tallest variety show the greatest increase in length and of the others in proportion to their dwarfism. It is noticeable that the response of isolated sections to GA is exactly the opposite of that of intact plants.

For a more thorough analysis of this phenomenon the compounds separately and all their combinations were tested. These data are given in Figure 7. It shows at first a noticeable division of the curves in two groups (at least in the dwarfs). Closer inspection reveals that the lower one is the group without IAA and that all curves with IAA are grouped on a higher level. This is an indication that the sections of the dwarfs respond more to IAA than to GA.

These curves furthermore show that:

- a. The response to GA separately is hardly more than that of the controls (buffer).

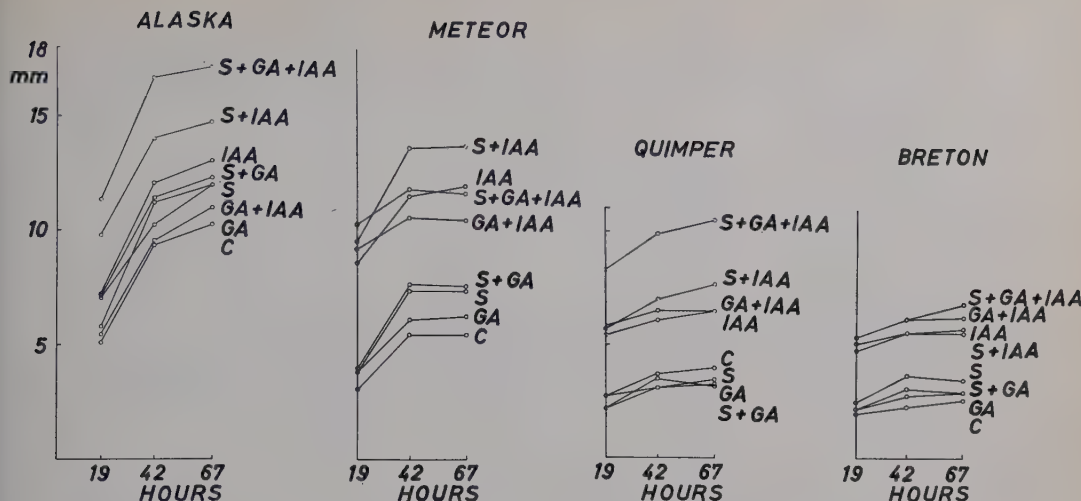


Figure 7. Increase in length of isolated sections of the four varieties in various combinations of sucrose, IAA and GA.

- b. The response to sucrose and to sucrose+GA is only slightly more than that of the controls.
- c. The response to IAA separately is more than that to GA separately, sucrose separately or GA+sucrose.
- d. Especially in *Alaska* and *Quimper* the response to S+GA+IAA is strongest.

These data seem to indicate that the response of the sections to the three compounds is determined more by IAA than by GA. That this is indeed true was proven as follows:

The component reactions in the total of S+IAA+GA was calculated, *viz.* the reaction to $(S+IAA+GA) - (IAA+GA) = S$ reaction.

$$(S+IAA+GA) - (S+IAA) = GA \text{ reaction.}$$

$$(S+IAA+GA) - (S+GA) = IAA \text{ reaction.}$$

The sum of the three reactions was put on 100 and the % of the separate reactions in the total drawn in a diagram (Figure 8). It shows that indeed the reaction to GA is small as compared with IAA. It furthermore shows that in the dwarfs the IAA part of the reaction is greater than in *Alaska*.

The general picture of figure 8 indicates that the response to GA is the same in all dwarfs and probably in *Alaska* somewhat stronger. This figure at least gives an explanation of the fact that the sections seem to respond to GA in another way as do the intact plants. *In the sections the bulk of the reaction is due to IAA whereas in intact plants the reaction is determined by GA.*

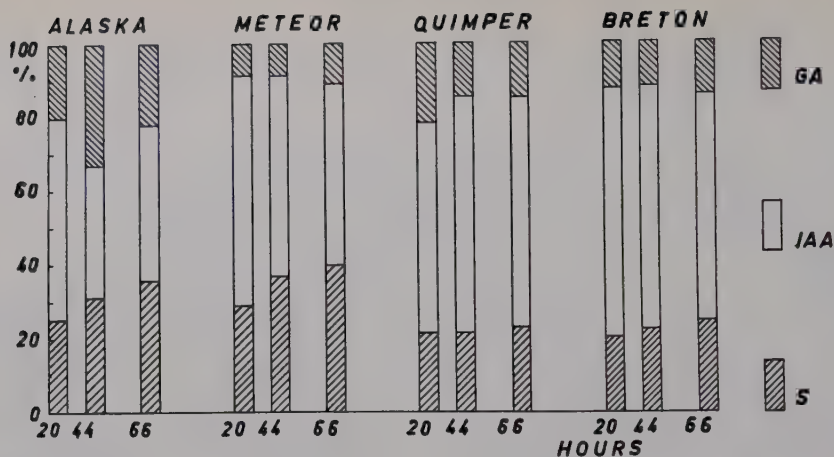


Figure 8. The part of sucrose (S), of IAA and of GA in the component reaction to S+IAA +GA. The total is put on 100 %. See further the text.

The questions arise whether or not the three compounds have synergistic activity or whether or not the reactions are additive. Both of these assumptions proved not to be true. None of the combinations, neither S+IAA or S+GA, nor IAA+GA is more active than the compounds when given separately.

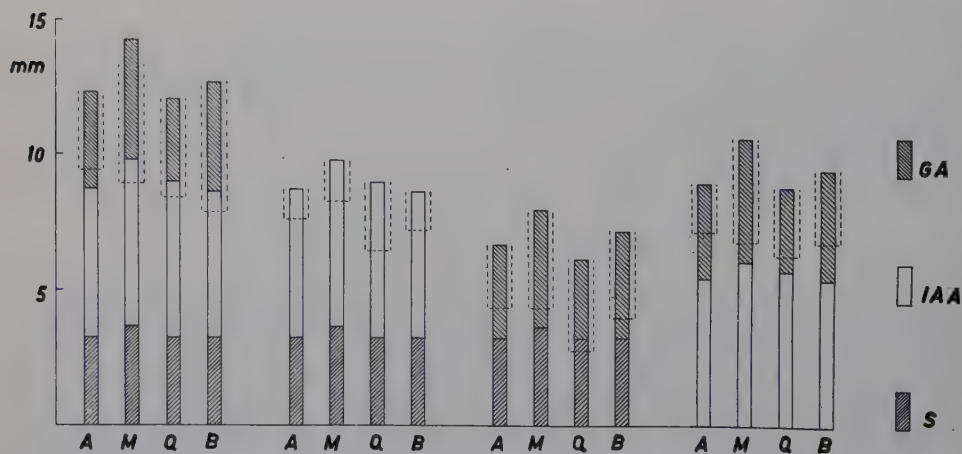


Figure 9. Increase in length of isolated sections in solutions of S, of IAA, and of GA in the combination of all three substances and in the combinations two by two. The columns indicated by the pointed lines represent the value which the sum of the separate reactions is more than the joint reaction.

Figure 9 shows this and proves that the reaction to
(S+IAA) is less than the sum of S and IAA;
(S+GA) is less than the sum of S and GA;
(IAA+GA) is less than the sum of IAA and GA;
(S+IAA+GA) is less than the sum of S and IAA and GA.

Some preliminary conclusions may be drawn from these data.

- a. In neither case do *Alaska* sections react differently from the dwarfs (except quantitatively).
- b. GA, instead of having additive or synergistic activity, looks as if it is antagonistic both with IAA and with S. However, an unknown limiting factor (s) might just as well be responsible for this apparent antagonism.

VI. Discussion

In the above mentioned experiments four varieties of peas were used, a tall ("normal") variety and three dwarfs. All four varieties when cultivated in darkness grow to the same size. When cultivated in light growth is reduced, in the dwarfs more than in the tall variety. Dwarfism, therefore, is the result of a growth reduction by light. When intact plants of the four varieties are supplied with GA the tall one does not respond, the dwarfs do respond and they all reach the same size as the tall pea. This implies that the smallest dwarf responds strongest.

When isolated internode sections of the same varieties are incubated in a GA solution, growth response is small. When, however, sucrose and IAA are added the response is considerable, provided the sections are from light grown plants and the reaction also takes place in light. Now the tallest variety responds strongest and the dwarfs less.

Considering that sections do not react in darkness, it seems logical to associate the GA activity with light. For intact dwarfs of which growth is determined by light intensity, the assumption can be made that GA reduces or removes the light inhibition. This is in close agreement with the fact that the dwarf which is inhibited most, is also the strongest responding to GA.

It remains to be investigated whether GA acts directly or indirectly and in which light reaction. Experiments along this line are being performed, among others, by Lockhart (1956, 1958), by Purves and Hillman (1958) and Hillman (1959).

For *isolated internode sections* the conclusion is obvious that in such small plant fragments only a slight light growth reduction is present which is completely removed by GA, after which the much stronger reaction to IAA determines growth. Only quantitative determinations of endogenous GA can give

a clue to the solution of this problem and also to the possibility that light inactivates endogenous GA, in the dwarfs more than in the normal variety.

Concerning the relation between GA and IAA activity, it may be observed that the substances have neither synergistic nor additive activity, but are rather antagonistic. In the present investigations this might be a consequence of supra optimal concentrations or of unknown limiting factors, but it also may turn out to be a general relation between GA and IAA.

VII. Summary

1. Four varieties of *Pisum sativum* were used, three dwarfs (*Meteor*, *Gloire de Quimper* and *Petit Breton*) and a "normal" tall variety (*Alaska*).

2. Dwarfism proved to be determined by light. Grown in darkness all varieties reach the same size. In light, growth is reduced, in the dwarfs more than in the tall variety.

3. In white and yellow light of equal incident energy level response is different. Growth reduction in *Alaska* is rectilinearly related to the logarithm of intensity in white light; in the dwarfs the sensitivity to this light is much higher than in *Alaska*.

In yellow light *Alaska* shows no growth reduction, no response at the higher intensities and a "yellow-etiolation" at the lower intensities. The dwarfs are reduced by all intensities.

3. GA counteracts the light reduction. Intact plants of the four varieties supplied with GA all grow to the same size.

5. Isolated internode sections of the four varieties respond very slightly to GA; addition of IAA and sucrose makes the response much stronger. In the complex reaction the response is due more to IAA than to GA.

6. No additive nor synergistic activity of GA with IAA or sucrose was found; always the response to the combined substances is less than the sum of the single reactions, the compounds are therefore rather antagonists, if not other unknown factors are limiting the reaction.

The author wishes to express her sincerest thanks to Dr. E. C. Wassink director of the Laboratory of Plant Physiological Research of the Agricultural University at Wageningen and his collaborator Ir. P. J. A. L. de Lint for placing at her disposal the technical equipments of this laboratory for growing plants under different light intensities and colours.

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Amide Metabolism in Wheat Leaves Infected With Stem Rust

By

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Introduction

The accumulation of glutamine in rust-infected plant tissues is a characteristic feature of the pathologically altered metabolism (Shaw 1957, Rohringer 1957, Montant 1959). A detailed study of this phenomenon seemed to be justified for more than one reason.

1. An increase in respiratory rate is a widespread phenomenon in host tissues infected by parasites (Allen 1953, Farkas and Király 1958, Uritani and Akazawa 1959, Rubin 1960 a). Despite of detailed studies carried out among others with rust infected wheat plants (Farkas and Király 1955, Samborski and Shaw 1956, Shaw and Samborski 1957, Király and Farkas 1957 a, b) the nature of respiratory stimulation is but little understood. It is generally accepted that the phosphate acceptor (ADP) level of the tissues is a major factor playing a role in the regulation of the overall respiratory rate. Consequently, as already suggested by one of us (Farkas 1957), the stimulated biosynthesis of glutamine in infected tissues might contribute to the respiratory increase by consuming ATP and enhancing the ADP level.

2. The accumulation of glutamine is apparently connected with alterations in the ammonia-metabolism of the attacked tissues. Excessive ammonia accumulation, as claimed by Suchorukov (1956), might play a role in rust resistance.

3. There seems to be some connection between glutamine accumulation and rust resistance. Samborski and Shaw (1957 a, b) have shown that treatment

with maleic hydrazide breaks the resistance of Khapli wheat to stem rust and the change of reaction type is associated with a marked increase in free amino acids, particularly glutamine. Other methods used by Forsyth and Samborski (1958) to change the host reaction from resistant to susceptible also led to a pronounced increase in soluble nitrogen the most abundant components of which are glutamic acid and glutamine.

4. Recent studies revealed the participation of glutamine in biosynthetic processes (biosynthesis of purines and chitin) which are evidently of vital importance for the development of the fungus (cf. Hartmann and Buchanan 1959, Gibbs 1959).

Materials and Methods

Wheat seedlings (varieties Little Club, Khapli and Vernal) were grown under ordinary greenhouse conditions, and the primary leaves were inoculated with a suspension of stem rust (*Puccinia graminis tritici*) uredospores (race 21 or 15 B). Symbols of reaction types of rust-infected wheat varieties are used throughout the text in accordance with Stakman (cf. Stakman *et al.* 1944). Leaves collected from both healthy and diseased plants 8—12 days after inoculation were used for the assays.

The respiration rates of small leaf-sections were measured at 30°C, using standard Warburg respirometer techniques.

The ammonia content of leaf tissues was assayed in deproteinized (alcoholic) extracts by the use of the micro-diffusion method.

The concentration of amino acids was determined by quantitative paper chromatography.

The activity of glutamine synthetase was assayed by two independent methods. The increase in glutamine concentration in crude extracts in the presence of added glutamic acid, NH_4Cl , ATP, cysteine and MgCl_2 was measured by quantitative paper chromatography. Alternatively, ammonium chloride was replaced by NH_2OH in the reaction system and the amount of glutamyl-hydroxamic acid produced was determined colorimetrically (Webster 1953, Loomis 1959).

Deamidase and deaminase activities were measured *in vivo* by infiltrating the substrates to be tested ($10^{-2} M$) into the leaves and measuring the amount of ammonia liberated after an incubation period of 16 hours.

Results

a. *Ammonia content of rusted tissues.* The ammonia content of rusted tissues was compared with that of healthy controls. The results are summarized in Table 1. It may be seen that the ammonia content is greatly increased upon infection. There was no clearcut difference between the ammonia production of susceptible and resistant combinations with the possible

Table 1. *The effect of rust infection on the ammonia content of wheat leaves.*

Variety	Infection type	NH ₃ concentration µg/g. fresh weight	
		Control	Infected
<i>T. compactum</i> "Little Club"	4	35	59
<i>T. dicoccum</i> "Khapli"	0;	27	35
<i>T. dicoccum</i> "Khapli"	1	29	50
<i>T. monoccum</i> "Einkorn"	0;	28	28
<i>T. timopheevi</i>	1	33	40

Infection with stem rust race No. 21.

The reaction type of Khapli wheat (0; and 1 respectively) depended upon the light regime and temperature prevailing during disease development.

exception of plants giving totally resistant reaction (infection type 0;), *i.e.* when no sporulation of the fungus took place at all. In this latter case no or little ammonia accumulation was observed.

b. *The effect of ammonium salts on the respiratory rate.* To test the eventual role of ammonia production in the parasitically stimulated respiration healthy and rusted wheat leaves (3×10 mm. pieces) were floated on the surface of 0.025 M NH₄NO₃ or NH₄Cl solutions for various periods. The determination of O₂-uptake indicated a pronounced increase after a treatment for 2 hours. The response was in most experiments less with infected plants, probably because of their higher endogenous ammonia content (Table 2). The stimulation with both healthy and infected tissues gradually disappeared in further 2—3 hours. These experiments favor the view that the increased ammonia production of infected plants might partly contribute to their higher respiratory rate. To support this idea we investigated in how far the other metabolic changes induced by ammonia-feeding are similar to those elicited by rust infection.

c. *Changes in soluble nitrogen compounds.* Feeding of healthy tissues with ammonium salts resulted in a considerable increase in glutamine concentration (Table 3). Higher glutamine content of rusted tissues was reported in-

Table 2. *Effect of feeding with ammonium salts on the respiratory rate of wheat leaves.*

Variety	QO ₂ -values			
	Healthy		Infected	
	Control	NH ₄ -treated	Control	NH ₄ -treated
<i>T. compactum</i> "Little Club"	2.5	3.3	5.0	5.4
<i>T. dicoccum</i> "Khapli"	2.8	3.6	—	—

Table 3. *Changes in soluble nitrogen compounds in wheat upon rust infection, treatment with ammonium salts or α -ketoglutarate.*

Variety	Treatment	Rust race	Reaction type	Concentration: mg/g. fresh weight					
				Glutamic acid		Glutamine		Asparagine	
				H	I	H	I	H	I
Little Club	H ₂ O	21	4	0.050	0.052	0.031	0.105	0.013	0.050
Khapli	H ₂ O	21	1	0.047	0.049	0.035	0.070	0.020	0.055
Vernal	H ₂ O	15B	3	0.058	0.065	0.027	0.060	0.009	0.050
Vernal	H ₂ O	21	0;	0.058	0.060	0.027	0.040	0.009	0.040
Little Club	NH ₄ NO ₃ 0.025 M	—	—	0.015	—	0.155	—	0.014	—
Little Club	α -ketoglutarate 0.01 M	—	—	0.152	—	0.040	—	0.015	—
Little Club	α -ketoglutarate + 0.025 M NH ₄ NO ₃	—	—	0.140	—	0.185	—	0.016	—

H=healthy.

I=infected.

dependently by Shaw (1957) and Rohringer (1957). Their findings, which were fully confirmed in our experience, indicate that the *major* change in the soluble nitrogen compounds in wheat leaves upon infection by rust fungi and upon treatment with ammonium salts is similar. On the other hand, the effect of ammonium salts and rust infection is not fully identical. Whereas (in contrast to Rohringer 1957) we observed an increase in asparagine in rusted tissues the asparagine level exhibited no change in healthy tissues upon feeding with ammonium salts. Furthermore, the marked decrease of glutamic acid in tissues treated with ammonium salts (Table 3) is in contrast with the unchanged level of glutamic acid in rusted wheat leaves. The possible explanation of this latter phenomenon is that in rusted leaves we are facing a "glutamic acid sparing" process because the activity of glutamic acid decarboxylase is dramatically decreased (Király and Farkas 1957 c, Waygood and Smith 1959).

The decrease of glutamate concentration in NH₄⁺-treated leaves is apparently due to the low speed of glutamate synthesis compared with its utilization (glutamine synthesis). A relatively low activity in healthy tissues of glutamic acid dehydrogenase (an enzyme which takes part in glutamate biosynthesis) compared with the high activity in affected tissues was demonstrated by Waygood and Smith (1959) for another rust disease. In our case, however, the sluggish glutamate synthesis in healthy leaves can not be explained by low glutamic acid dehydrogenase activity since the feeding of α -ketoglutarate to the tissues resulted in the rapid accumulation of high amounts of glutamate (Table 3). Apparently the α -ketoglutarate concentra-

tion is the limiting factor. Strangely enough, the glutamic acid synthesized from the externally supplied α -ketoglutarate is but slowly transformed into glutamine. High amounts of glutamic acid accumulate in the tissues treated with α -ketoglutarate for 10—16 hours, without a concomitant increase in glutamine concentration (Table 3).

d. *The accumulation of amides and disease resistance.* The accumulation of glutamine in infected susceptible tissues is beyond any doubt (cf. Shaw 1957, Rohringer 1957 and the present results). The data concerning wheat-rust complexes of the resistant type are, however, contradictory. Shaw (1957) does not refer to studies on resistant combinations. According to Rohringer (1957) no glutamine or asparagine accumulates in infected resistant wheat varieties. Our present studies indicated that both susceptible and resistant wheat-rust complexes accumulate glutamine and asparagine, although the amount of glutamine accumulated is less in resistant tissues. This is clearly shown by the experiments with the wheat variety Vernal (Table 3). This wheat gives resistant reaction (type 1) to infection with stem rust race 21 and susceptible reaction (type 4) if infected with race 15 B.

e. *The activity of glutamine synthetase.* The characteristic increase in glutamine concentration of rusted tissues might be due to their increased ammonia level but the stimulation of glutamine synthetase might also play an equally important role. The assays of glutamine synthetase in crude extracts gave lower values than reported by previous authors working with purified preparations (Webster 1953, Loomis 1959). However, it was clearly established that the activity of glutamine synthetase is higher in the infected tissues.

f. *Deamidase and deaminase activity of rusted tissues.* The higher ammonia level in infected tissues might be due to the increased activity of deamidases or deaminases. To test this idea 10^{-2} M solutions of various amino acids, glutamine and asparagine were infiltrated into healthy and infected wheat leaves, the excess of water was evaporated, and the leaves were placed in 10^{-2} M solutions of the substances to be tested. After an incubation period of 12—16 hours the ammonia content of the leaves was measured. Results are summarized in Table 4. It can be seen that the deamidase activity of rusted leaves is tremendously increased. On the contrary, in healthy leaves little or no ammonia was liberated from the amino acids tested.

g. *Deamidase activity of germinating uredospores.* 5 mg. of stem rust uredospores were incubated in Conway units with 10^{-2} M asparagine. To get the spores germinated under self-inhibited conditions (closed vessels) 10^{-4} M pelargonaldehyde was added to the solution (cf. Farkas and Ledingham 1959). Controls were germinated in vessels containing 10^{-4} M pelargonaldehyde only. After an incubation period of 14—16 hours the spores were elimi-

Table 4. *Deamidase and deaminase activity of wheat leaves infected with stem rust.*

Infiltrated compound 10 ⁻² M	NH ₃ liberated, expressed in µg/g. fresh weight	
	Healthy	Infected
Glutamine	30	270
Asparagine	7	230
Glutamic acid ...	— 7	0
Aspartic acid ...	0	32
Tryptophane ...	0	28
Serine	0	0

Infiltration of glutamic acid resulted in a slight decrease of the endogenous ammonia concentration.

nated by filtration and the NH₄⁺-ion content of the filtrate was determined. During the incubation period the germinating spores liberated several hundred µg. ammonia from the asparagine added as substrate. Therefore, the developing fungus seems to be responsible for the extremely high deamidase activity of the infected tissues.

Discussion

The observations that (a) the ammonia concentration in the diseased tissues is increased, (b) the respiration of healthy leaves can be increased by the addition of ammonium salts, (c) the respiration of infected leaves can be raised by ammonium salts only to a lesser extent, (d) the treatment with ammonium salts results in the tissues in metabolic changes similar to those elicited by infection (accumulation of amides), justify the conclusion that the parasitically produced ammonia might partly be responsible for the respiratory increase of the host tissues. However, the data clearly indicate that *only a fraction* of the disease-induced respiration might be attributed to ammonia accumulation. The stimulation is apparently due to a higher ADP level created by ATP consuming reactions like the NH₃-dependent glutamine and asparagine biosynthesis and probably also by an increased protein synthesis. In addition to the enhanced NH₃-production the increased glutamine synthesis of infected tissues is explained by their higher glutamine synthetase activity. Feeding experiments indicated that the low concentration of α-ketoglutarate and not the low endogenous ammonia level is the limiting factor of glutamic acid biosynthesis. Glutamic acid is namely readily synthesized from α-ketoglutarate without added ammonia. Therefore, the endogenous ammonia level is sufficient for the reductive amination of considerable amounts of

α -ketoglutarate. Strangely enough, the glutamate produced is but sluggishly metabolized further (*i.e.* converted into glutamine). One wonders why part of the endogenous ammonia is not used, or used only to a very slight extent, for the amination the glutamate formed during the α -ketoglutarate treatment. Externally supplied ammonia will namely immediately initiate an intense transformation of glutamic acid formed from added α -ketoglutarate. It seems that the internal ammonia source is preferentially used for glutamic acid synthesis whereas the externally supplied ammonia is mainly used for glutamine synthesis. This observation raises the idea of "compartmentization" of various steps (components) of glutamine biosynthesis within the cell. In this respect the ammonia supplied externally behaves like the parasitically produced ammonia.

Compartmentization of the processes of amide metabolism is also indicated by our studies on the source of ammonia accumulating in diseased tissues. The finding was extremely puzzling that amides introduced into the infected leaves are broken down despite the ability of these tissues to accumulate glutamine and asparagine from endogenous sources. The amides synthesized in the tissues are evidently protected from deamidation, probably by compartmentization.

Because of the apparent controversy discussed above it is very difficult to solve the problem of the origin of ammonia produced in diseased tissues. The most plausible explanation, the extremely high deamidase activity of infected tissues and germinating spores is more or less ruled out because amides do accumulate in the tissues. However, some deaminase activity was also observed and the increased polyphenoloxidase activity of infected tissues (Király 1958) might also be connected with ammonia production via the oxidative deamination of amino acids in the presence of polyphenols and polyphenoloxidase (Hess 1958).

Only a few suggestions can be made as to the role in rust resistance of the metabolic alterations described. The high ammonia content of infected wheat leaves is not in correlation with disease resistance. On the contrary, the abundant growth of the parasite seems to be associated with intense ammonia production. It must be stressed, however, that the above statement is apparently valid only for obligate parasites. The Moscow-group (Rubin and Ivanova 1959, Rubin 1960 b) observed accumulation of ammonia in infected cabbage tissues resistant to *Botrytis cinerea*. The difference between the wheat-rust and cabbage-*Botrytis* complexes is also indicated by the higher level of parasitically stimulated O_2 -uptake in the resistant cabbage tissue in contrast to the markedly higher respiratory increase in rust-infected susceptible wheats (Samborski and Shaw 1956, Király and Farkas 1957 b).

We do not know for the present whether or not the increased glutamine level in susceptible rust-infected tissues has any benefit for the parasite. However, glutamine is a precursor of substances of evidently vital importance for the rust fungus. Thus the participation of glutamine in purine biosynthesis (Hartmann and Buchanan 1959) and the involvement of glutamine or ammonia in chitin biosynthesis (cf. Gibbs 1959) is an established fact. Allen (1959) pointed out the close connection between the respiratory increase, chitin biosynthesis and rust development in infected susceptible wheat. The idea is tempting that the enhanced production of ammonia and glutamine has also some role in both the respiratory stimulation and chitin biosynthesis in the infected tissues.

Summary

Wheat leaves infected by stem rust exhibit a high respiratory rate and accumulate ammonia, glutamine and asparagine. Feeding of ammonium salts to healthy tissues led to metabolic alterations which are highly similar to those elicited by rust infection: the respiratory rate was enhanced and glutamine accumulated. The response of rusted leaves to treatment with ammonia was less. The extent of parasitically increased glutamine accumulation is dependent on the reaction type of the tissues. Less glutamine is synthesized by resistant combinations.

The mechanism of the parasitically stimulated glutamine synthesis was studied. It has been found that in addition to the increased ammonia production by infected leaves the parasitically enhanced activity of glutamine synthetase also contributes to the accumulation of glutamine. Strangely enough, parallel with glutamine and asparagine accumulation the ability of infected tissues to deamidate externally supplied amides is enormously increased. Slight activation of some deaminases was also observed.

Germinating stem rust uredospores exhibited a high deamidase activity. Therefore, the enhanced deamidase activity of the infected tissues might be attributed to the developing fungus. The problem of simultaneous activation of amide synthesis from endogenous sources or from externally supplied ammonium salts and the enormous stimulation of deamidase activity in rusted tissues is discussed and as an explanation the compartmentization of the processes of nitrogen metabolism is offered.

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Growth Action of EDTA in Light and Darkness

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The influence of chelating agents on growth has been dealt with in about twenty papers since Bennet-Clark in 1956 suggested a growth-regulating mechanism of EDTA, and Heath and Clark presented evidences of a similarity between chelating agents and auxins. It can hardly be denied that EDTA exerts a pronounced growth effect under certain circumstances, nor that most papers on this subject are obviously preliminary in nature. The idea launched by Bennet-Clark was that EDTA acts by chelating Ca from the cell wall, an assumption still discussed by Heath and Clark in 1960. However, it was shown already in 1957 that EDTA inhibits strongly the cell divisions in root meristems, which is not the primary action of either auxins or Ca at deficiency (Burström and Tullin), and in three papers in 1959 Carr and Ng claimed that the Ca-hypothesis is untenable. Finally, in two papers in 1960 it was shown that EDTA reduces the root meristem activity even in the presence of a 400 : 1 excess of Ca (Burström), and that auxins do not remove Ca from cell walls either (Cleland).

This seems to clarify the situation as far as the Ca-hypothesis is concerned, leaving an open field for another approach to the study of the mechanism of the chelate action. However, it must be considered that chelation of metals is an unspecific kind of binding, all chelating agents theoretically binding metals in the same sequence of stability (Martell and Calvin 1952). Thus it is not certain that there is one common explanation of all growth effects even by one chelating agent.

It was found in an preliminary experiment (Burström 1960) that an EDTA-inhibition of wheat root growth (*a*) is restricted to darkness but absent in

light, and (b) that it can be reversed by an addition of Fe^{3+} . Since Fe also was found to be a pre-requisite for a light inhibition of growth it seems to suggest that Fe^{3+} is the active metal removed by EDTA from the roots.

Such an assumption may be premature. *A reversal of an EDTA action by a metal does not mean that this metal must be the physiologically active one; it might only release another metal, which had been chelated from the tissue.* This general objection makes it difficult conclusively to prove what metal is the physiologically active one, the more so as a similar exchange may occur on endogenous chelate formers, which are abundantly present in the cell (cf. Martell and Calvin on natural chelate ligands).

An attempt has been made to study the problem of the EDTA-inhibition of root growth by combining it with all metals which may possibly occur in the tissues. Martell and Calvin have given the order of complex formation as:



Of these metals Ca and Mg have been constantly present in the nutrient solutions of our experiments in an excess of at least 200 : 1 over the EDTA added. They can be definitively disregarded from the chelate picture. The interpretation of the mode of action of the metals is further complicated by the fact that beside chelating EDTA and replacing other ions, all of them except Ni may exert intrinsic physiological, and all of them poisonous effects. A tolerably complete investigation of the metal-EDTA interactions in growth would thus require all possible combinations of different metals in order to eliminate nutrient deficiencies, together with EDTA in varying additions and in light and darkness. This is practically unfeasible. Short-cuts had to be made, and the experiments were planned in order to test the working hypothesis that EDTA inhibits growth by chelating iron.

Methods

The experimental methods are the same as those described in Burström (1960). In the basal solution Fe enters as $\text{Fe}^{\text{III}}\text{EDTA}$. Growth was studied in seven-day-tests on cultures of excised wheat roots. Growth was measured and will be discussed in terms of:

- (a) the number of cells formed in the longitudinal direction during seven days, which is a relative measure of the cell multiplication in the apical meristem,
- (b) the final cell length attained, which is usually correlated with
- (c) the true rate of cell elongation over time, which was determined in some critical instances only.

In some experiments with very low concentrations of added metals the solutions were renewed after three or four days of growth. It is known that wheat roots are

Table 1. *Effect of renewal of the nutrient solution on the growth. Two examples.*

Time and treatment	A		B	
	cell length μ	cell number	cell length μ	cell number
		per day		per day
First 4 days	180	42	186	41
last 3 days no renewal	160	48	173	49
last 3 days solution renewed	153	44	160	46
Over 7 days		per 7 days		per 7 days
no renewal	171	311	180	310
solution renewed	168	300	174	299

sensitive to subculturing (Almestrand 1957) and it was checked that under these conditions the shock to the roots from the transference did not significantly alter their growth (Table 1). As often observed the final cell lengths decrease slowly during the seven days, which is accounted for in the measurements.

The EDTA was added as the di-sodium salt; unless otherwise stated this and the heavy metals as chlorides or sulphates were always given in 10^{-6} M concentration.

The significance of the determinations of cell lengths and cell numbers require some comments. Computed according to the least square method a difference in 15μ in cell length and in cell number of 40 should be regarded as significant. However, the real accuracy must be greater judging from the reproducibility of the results. One typical example is given in Table 2. The directions of the responses are those always obtained with Fe and light, all being significant except differences in cell lengths without Fe (A—C) and in cell numbers in light (A—B) and dark (C—D), respectively. However, cell lengths are usually reproducible with smaller differences in average values than 5μ , and cell numbers with less than 10, which is better than expected from the mean errors. The reason is that the variations in the material, although numerically large, are very regular. The ordinary probability calculations then fail.

Roots of average size and shape were selected for the photographs.

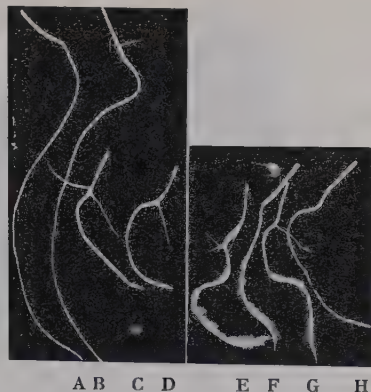
General Action of EDTA and Light

A number of experiments have unanimously confirmed the preliminary observation that EDTA strongly reduces the cell multiplication in darkness

Table 2. *The action of Fe in light and darkness with computations of mean errors according to the least square method. Basal solution without Fe. FeCl₂ added in 10^{-6} M.*

Treatment	Root growth mm.	Cell length μ	Cell number
A. Light — Fe	34.4 ± 1.7	168 ± 3.2	205 ± 11
B. + Fe	25.0 ± 1.1	125 ± 2.9	200 ± 10
C. Dark — Fe	60.0 ± 2.7	167 ± 3.9	359 ± 18
D. + Fe	66.6 ± 2.3	181 ± 3.7	368 ± 15

Figure 1. *The morphology of EDTA-treated roots in light and darkness. Basal solution +Fe 10^{-6} , +Mn 10^{-8} , +Zn 10^{-8} . — Note the profuse root hair formation in light. — Natural size.*



Treatment				Cell length	Cell number
A	Darkness	EDTA	0	170	306
B			10^{-6}	180	256
C			$3 \cdot 10^{-6}$	141	126
D			10^{-5}	177	75
E	Light		0	116	198
F			10^{-6}	111	188
G			$3 \cdot 10^{-6}$	89	241
H			10^{-5}	102	213

but not in light, and that the cell length is less affected. This is exemplified in Figures 1, 2, 4, 11. The experiments also show the strong reductions of the cell divisions by light, irrespective of EDTA and metal additions, from values around 300 to about 200. As discussed in the previous communication (Burström 1960) light inhibits cell elongation only in the presence of Fe (see further Figures 1, 2, 3, 5 and Tables 5, 6, 8).

The striking morphology of the EDTA-treated roots is illustrated in Figure 1. It should especially be observed that with high EDTA-additions the over-all growth and the rate of cell multiplication in the light exceed that in the dark. This eliminates one possibility of explaining the connexion between light and EDTA, namely that both inhibit the same reaction. If it were inhibited by one factor, the other could lack substrate for a further effect. This obviously does not hold true. The EDTA-effect actually disappears in light.

The picture is almost the same with Fe absent (Figure 2). There is a significant effect of EDTA at the highest addition in light, but the dark-grown roots are at least ten times more sensitive. The inhibition is fully reversed by Fe^{III} , and the cell division rate with EDTA+Fe will even surpass that of the control (Table 3). It has been emphasized that EDTA does not appreciably affect the cell length, but it does, as shown in the table, considerably

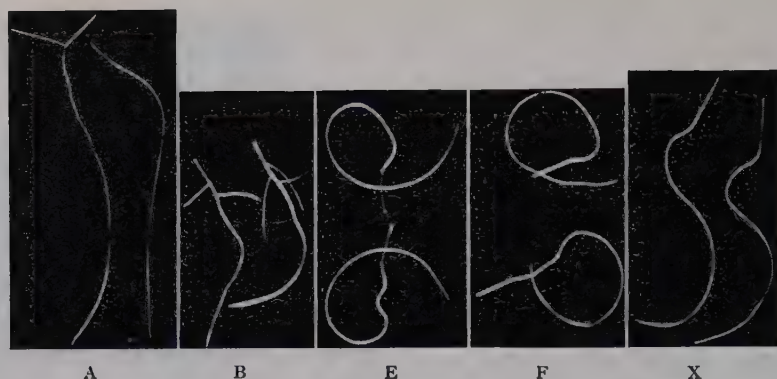


Figure 2. *The growth shape of roots in light and darkness. Basal solution without Fe.* — A, B, E, and F: Roots in natural position when taken from the culture flasks; those from A were curved along the wall of the flask. Note the regular spiral growth in E and F. X=roots from E stretched as far as possible (cf. root E in Figure 1). Two roots of each treatment. Natural size.

Treatment				Cell length	Cell number
A	Darkness	control		166	261
B		EDTA	10^{-6}	131	117
C			$3 \cdot 10^{-6}$	153	94
D			10^{-5}	148	107
E	Light	control		181	191
F		EDTA	10^{-6}	165	193
G			$3 \cdot 10^{-6}$	152	162
H			10^{-5}	167	126

hamper the rate of elongation, which is independent of both cell multiplication and final cell length. This effect is likewise reversed by Fe.

It has been shown with EDTA concentrations down to 10^{-9} M (Figure 3) that no positive effects are obtained and thus presumably no detoxication of heavy metals (cf. Heath and Clark 1960).

However, such experiments are not at all conclusive. They must be carried out with Fe, Mn, and Zn absent, or the metals would bind the small amounts of EDTA added, but then, on the other hand, a deficiency of these elements preventing a growth promotion cannot be excluded.

Figure 2 illustrates a general property of the roots, which we have not found described in the literature. In light the roots grow in a regular, wide spiral, usually in the same direction and of almost the same diameter, 8 to 10 mm. With due respect to the fact that roots grow upside-down, the spiral usually has the same counter-clockwise (left-hand) direction as is common in winding stems. One cause may be that the roots cannot respond to gravity in the normal way but are forced to grow horizontally. On the other hand,

Table 3. *The reversal of the EDTA-inhibition in darkness by FeCl₃. — Basal solution +Mn +Zn. Maximal rate of cell elongation has been estimated graphically from cell elongation curves.*

Na ₂ EDTA	FeCl ₃	cell length μ	cell number	maximal rate of elongation μ/h
0	0	184	246	23
10 ⁻⁶	0	186	113	9
10 ⁻⁶	3 · 10 ⁻⁷	146	194	9
10 ⁻⁶	10 ⁻⁶	165	295	24
10 ⁻⁶	2 · 10 ⁻⁶	183	275	25
10 ⁻⁶	2.5 · 10 ⁻⁶	180	271	27

the fairly regular spiralization points to some endogenous property; the result may depend upon nutation growth (cf. Baillaud 1957) combined with the coercive, geotropic position. A connexion with the rotation sensitivity of oat roots (Jones 1960) may be worthy of attention.

The next step was to compare the ability of different metal ions to reverse the EDTA inhibition. This was done under the following assumptions, (a) that EDTA chelates from the root some indispensable metal, (b) that other ions may release this metal from EDTA, and (c) that the order of activity will give a clue to the identity of the endogenous metal. If it was a question of an ideal chelation only, the activities of the metals should decrease in the order Fe^{III}—Cu—Ni—Co—Zn—Fe^{II}—Mn—Ca—Mg. Those to the right of the endogenous metal should give little or no response, but the endogenous metal — if present in this series — the highest activity.

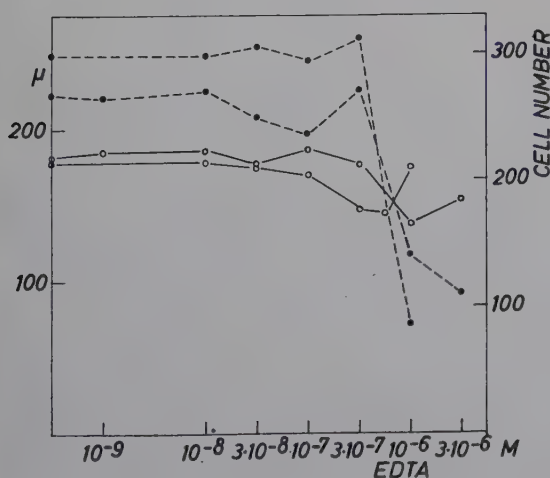


Figure 3. *The influence of Na₂EDTA in varying concentrations on the cell elongation and cell multiplication. Darkness. Basal solution —Fe, —Mn, —Zn. Two experiments. ○—— cell length, ●--- cell number.*

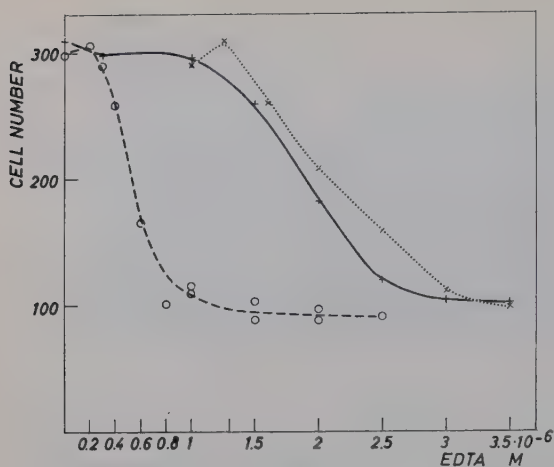


Figure 4. The influence of Na_2EDTA in high concentrations on the cell multiplication in the presence and absence of $\text{Fe } 10^{-6} \text{ M}$. Darkness. O---- no Fe, +—— FeCl_3 , ×---- $\text{Fe}^{\text{III}}\text{EDTA}$.

A quantitative estimation of the relative amount of endogenous metal chelated by EDTA can be made from concentration curves such as those in Figure 4. The concentration-activity curves for EDTA have nearly the ideal shape of titration curves, and the cell multiplication regularly decreases from ca. 300 to ca. 100 cells. It can be deduced from the left-hand curve, without addition of Fe, that an addition of about $1.1 \cdot 10^{-6} \text{ M}$ EDTA is required for a complete binding of the presumed endogenous metal.

Reversal of the EDTA-Inhibition

This was studied with six elements in eight independent series, for which average values are given in Table 4. All metals have been added in 10^{-6} M , equivalent to the EDTA. Tests were made in light and darkness, with and without an addition of $\text{Fe}^{\text{III}}\text{EDTA } 10^{-6} \text{ M}$. The reason for this was the presumption that Fe^{III} is involved in the growth effect, and the assumption that lack of Fe may cause Fe-deficiency interfering with the actions of other metals.

NH_4 -molybdate was included only in order to round off the list of micro-nutrient elements — boron was out of the question; Mo gave as expected no response of interest. A response has been judged to be significant if it has both met with statistical requirements and shown good agreement in independent series.

The most striking result is that roots without Fe in light do not respond either to EDTA or to the metals, except with a toxicity of Cu. Since these roots grow and behave normally the indispensable amounts of heavy metals must be so firmly bound that they cannot be chelated by EDTA or antago-

Table 4. *The inhibition of rooth growth by Na₂-EDTA and its reversal by metals. All concentrations 10⁻⁶ M. Fe added as Fe^{III}-EDTA, Mn as chloride, Mo as molybdate, all others as sulphates. Control solution without heavy metals. Significant inhibitions in italics, promotions in bold types, for EDTA as difference from control, for metals as differences from EDTA.*

Addition 10 ⁻⁶ M	Cell length μ				Cell number			
	light		dark		light		dark	
	- Fe	+ Fe	- Fe	+ Fe	- Fe	+ Fe	- Fe	+ Fe
Control	150	124	170	153	202	203	347	320
EDTA	145	136	<i>153</i>	<i>137</i>	192	208	<i>142</i>	<i>203</i>
+ Cu ^I	149	171	180	173	99	<i>160</i>	<i>94</i>	212
+ Ni	151	137	174	191	202	192	279	279
+ Co	151	175	144	150	227	206	200	219
+ Zn	131	140	166	146	216	207	263	323
+ Mn	133	153	157	180	175	184	167	251
+ Mo	133	130	146	130	179	215	149	255

¹ Tendency towards wavy shape of epidermis indicating inhibition of growth in the interior (note reduction in cell number) and continued elongation of surface layer.

nized by other heavy metals. This contrasts to the labile conditions in darkness.

The order of activity is not the one expected, not even for the cell division promotion in the dark. Zn and Ni are strongly active, Co and Mn less so or inactive, Cu falls out of the series entirely. The effects on the cell lengths are still more confusing: strong positive effects of Cu, Co, and Mn in the presence of Fe, but not of Zn, and by Ni exclusively in the dark. This does not resemble a series due to chelation stability. It is likely that the results are complicated by accessory nutritional and toxic effects, which necessitates a scrutiny of each metal separately.

The effect of *ferri-iron* on the cell elongation was comprehensively dealt with in the previous communication (Burström 1960). The inhibition in light and promotion in darkness are again exemplified (Figures 5, 6 and Tables 8, 9), as well as the negligible effect on the cell multiplication with no additional EDTA. Table 3 and Figure 6 show the reversal of the EDTA-inhibition of cell multiplication by Fe^{III} as EDTA-salt and chloride, as well as the lacking or small effect in the light. These results stand out clearly from a large number of experiments of different designs. The reversal does not prove that Fe is the endogenous metal since Fe^{III} is the strongest chelate-former of the metals of interest.

The EDTA-concentration curves in the presence of Fe^{III} (Figure 4) permit some quantitative considerations. It can be computed graphically that with FeCl₃ about $2.1 \cdot 10^{-6}$ M, EDTA gives a full inhibition. With $1.1 \cdot 10^{-6}$ M corresponding to endo-

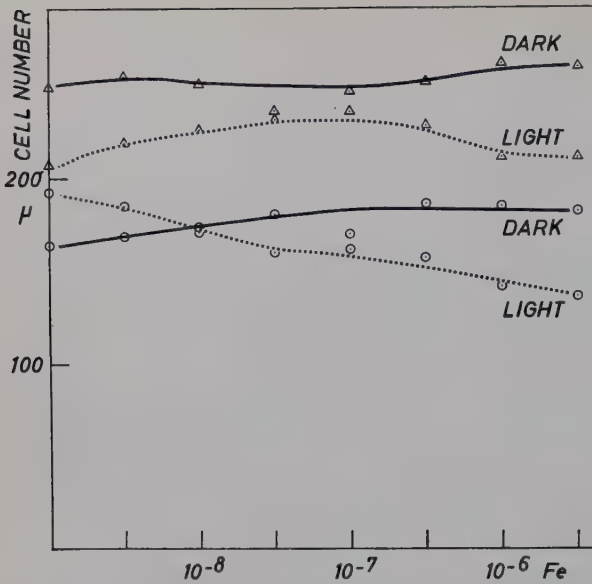


Figure 5. Influence of iron in light and darkness. Fe as Fe^{III} EDTA. ○ cell length, Δ cell number. Average of three experiments.

genous metal subtracted, the remaining 10^{-6} M tally with the amount of Fe added. With a constant addition of 10^{-6} M Fe^{III} EDTA, however, the same inhibition requires about $1.7 \cdot 10^{-6}$ M additional EDTA, leaving about 0.6 M unaccounted for. The computation is rather rough, but the main point is that Fe^{III} EDTA is less inhibitory than $\text{FeCl}_3 + \text{EDTA}$. The difference is too large to be neglected, and the question will be taken up in the discussion.

Figure 6. The influence of Fe on the cell elongation and cell multiplication in the presence of Na_2EDTA in light and darkness. Fe added as Fe^{III} EDTA in excess over a constant addition of Na_2EDTA 10^{-6} M. Values for treatments without Na_2EDTA and without Fe are indicated on the ordinates for cell length (left) and cell number (right) by the number of the corresponding curves. 1=darkness, Zn constant 10^{-7} M, 2=darkness Zn 10^{-8} M, 3=light Zn 10^{-7} M. Full-drawn curves cell length, broken curves cell number.

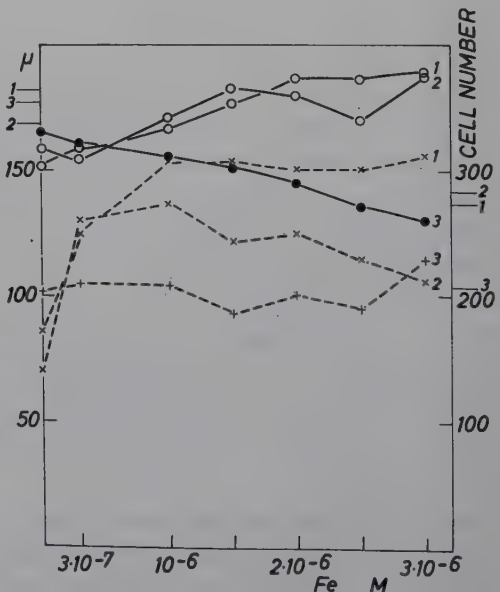
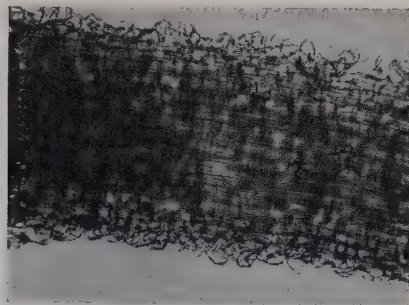


Figure 7. The typical structure of a root grown in a complete basal solution with $\text{Ni } 10^{-5} \text{ M}$. Note shedding of the spherical or sausage shaped epidermal cells.



Copper is distinguished by large cell division inhibitions due to a toxic effect probably also responsible for the irregular cell elongation noted in Table 4. The morphology at strong inhibitions is depicted in Figure 7. It resembles that of EDTA-treated roots described by Cormack (1959) or the roots with 'sausage' epidermis caused by certain auxinic inhibitors according to Hansen (1954). The phenomena must not be related, but could depend upon a similar pattern of localization of the inhibitions.

The Cu-inhibitions are absent or slight with Fe present. This cannot be due to an exchange of Fe for Cu in the EDTA but must depend upon a com-

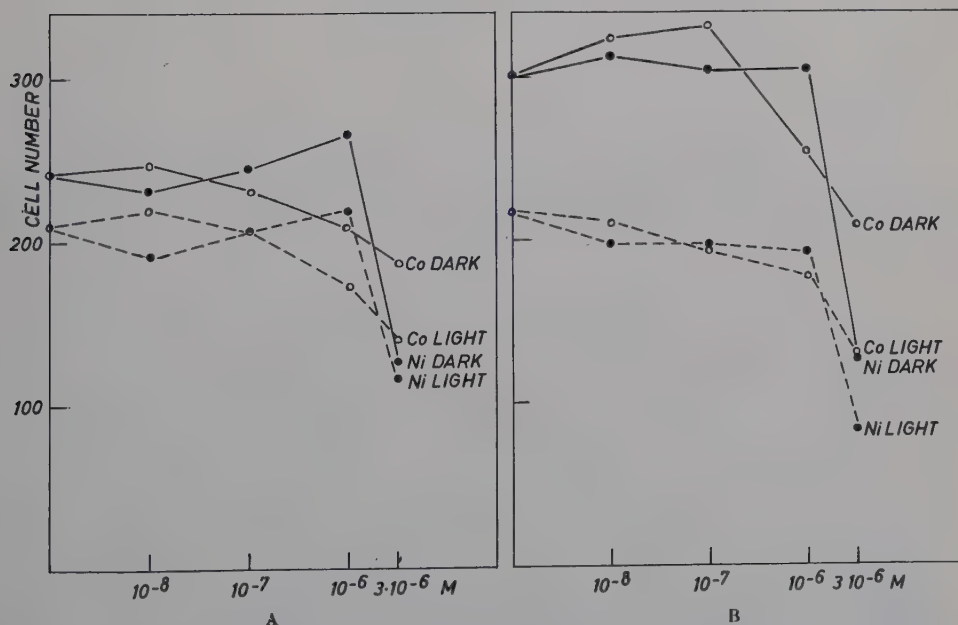


Figure 8. The influence of Ni and Co in varying concentrations on the cell multiplication in light and darkness. A with EDTA 10^{-6} M , B no EDTA.

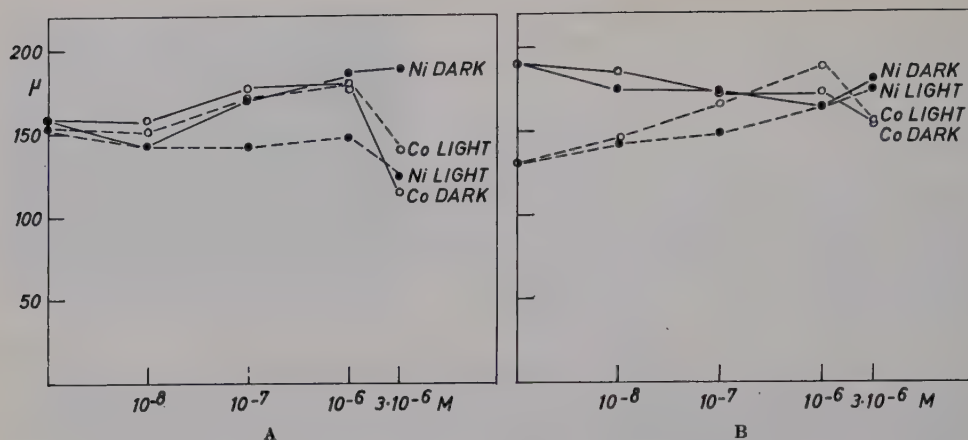


Figure 9. The influence of Ni and Co in varying concentrations on the cell elongation in light and darkness. A with EDTA 10^{-6} M, B no EDTA.

petition in some endogenous system. This toxicity may conceal a competition for EDTA.

Special attention has been paid to *nickel* and *cobalt*, because they have been applied as growth promoters in standard tests on auxins (Miller 1951, 1952, 1954, Thimann 1956, Busse 1959). Different explanations have been proposed (Thimann, Salisbury 1959, Busse) of the growth activity of Co, but only that of Busse seems to be supported by convincing experimental evidences.

The present material is confusing insofar as the chemically closely related Ni and Co differ markedly in their actions. Nickel reverses the EDTA-inhibition of cell multiplication (Table 4), Co slightly or not at all. According to Figure 8 this is due to a different type of inhibition by Co and Ni;

Table 5. The influence of Co and Ni 10^{-6} M on the cell elongation in light and darkness. Average of 4 series of experiments.

Treatment	Cell length μ					
	with FeIII-EDTA 10^{-6}			without Fe		
	light	dark	light effect	light	dark	light effect
Control	146	187	- 41	170	173	- 3
Co 10^{-6}	196	155	+ 41	166	158	+ 8
Control	136	189	- 53	180	180	0
Ni 10^{-6}	172	161	+ 11	132	178	- 46
Effect of Co	+ 50	- 32	×	- 4	- 15	×
„ „ Ni	+ 36	- 28	×	- 48	- 2	×

A B C D E F



Figure 10. *The nickel toxicity in light and darkness.* Basal solution without Fe. Note that no EDTA is present. — Natural size.

Treatment			Cell length	Cell number
A	Darkness	control	168	314
B		Ni 10^{-6}	170	274
C		FeCl ₃ 10^{-6}	192	294
D		Ni+Fe	155	290
E	Light	control	187	231
F		Ni 10^{-6}	113	144
G		FeCl ₃ 10^{-6}	156	207
H		Ni+Fe	143	141

the roots are more sensitive to low concentrations of Co than of Ni. It should be observed that the material of Figures 8 and 9 differs from that of Table 4 with regard to the presence of Mn, which alters the growth with Ni and Co absent. The difference between Co and Ni cannot depend upon a stronger reversal of the EDTA action by Ni, since it appears also in its absence (Figure 8 B), but endogenous competition may be involved.

The surprising effect on the cell length is that the EDTA-action is reversed by Ni in the dark irrespective of Fe-additions, but with Co in the presence of Fe both in light and darkness. This seems to depend partly on interactions with other indispensable ions, because the responses are more similar with both Fe, Mn and Zn present (Table 5). Both metals then increase the cell length in light and decrease it in darkness. A specific Ni-inhibition is restricted to light (Figure 10). The Ni and Co effects in a complete nutrient solution have been carefully studied. They are regulated by the presence of external Fe (Table 6) and are the reversal of the Fe-action. This was shown to depend upon a change in the rate of elongation (Burström 1960) and the same holds good for Ni and Co (Figures 11, 12). The metals reverse the ac-

Table 6. *The interaction between Co and Fe. Fe as Fe^{III}-EDTA, Co as sulphate, both 10⁻⁶ M. Significant responses especially tabulated.*

Treatment	Growth mm.	Cell length μ	Cell number	Significant effects on			
				cell length by		cell number by	
				Fe	Co	Fe	Co
<i>Light</i>							
Control	32.6	157	207				
Co	34.4	152	226				
Fe	19.0	103	185	— 54			
Co + Fe.....	33.9	171	198	+ 19	+ 68		
<i>Darkness</i>							
control	51.1	133	384				
Co	39.7	119	334		— 14		— 50
Fe	55.1	181	305	+ 48		— 79	
Co + Fe.....	45.9	153	302	+ 34	— 28		

tion of light and have both the opposite action as Fe. They are also inactive in the absence of Fe.

The effects of *zinc* in the presence of EDTA are simple (Table 4): no effect on cell length, no effect on cell multiplication in light, but an efficient

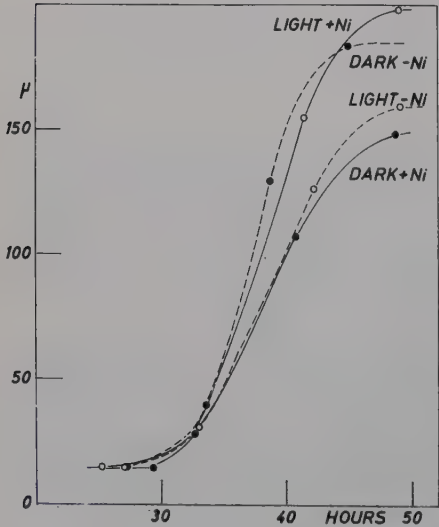


Fig. 11.

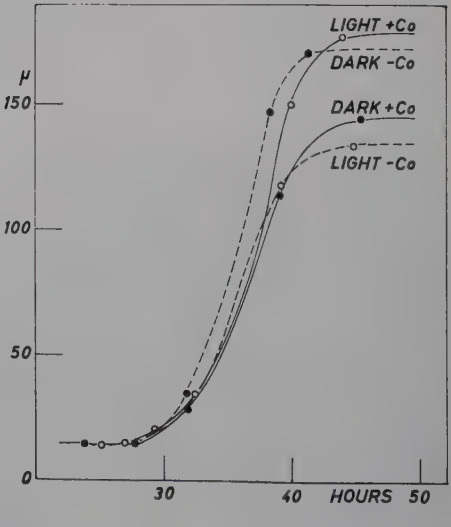


Fig. 12.

Figure 11. *The influence of Ni 10⁻⁶ M on the time course of the cell elongation in light and darkness.*

Figure 12. *The influence of Co 10⁻⁶ M on the time course of the cell elongation in light and darkness.*

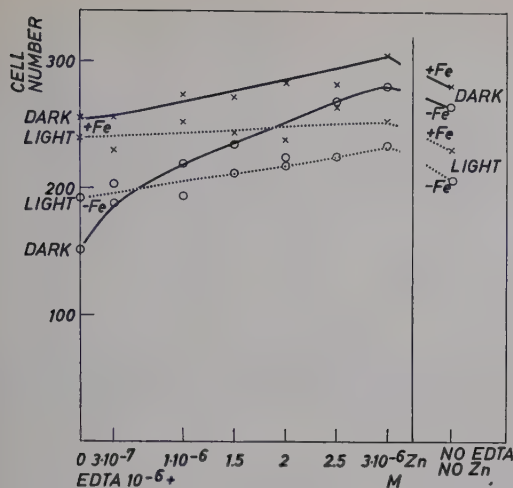


Figure 13. The reversal of the EDTA-inhibition of cell multiplication by Zn. Basal solution with Mn, with or without $\text{Fe}^{\text{III}}\text{EDTA } 10^{-6} \text{ M}$ and $\text{Na}_2\text{EDTA } 10^{-6} \text{ M}$. Zn varied. To the extreme right a control without both Na_2EDTA and Zn.

reversal of the inhibition in darkness. This is also depicted in Figure 13 with almost the same results; the differences are due to the presence of Mn in the basal solution.

Figure 13 illustrates the striking EDTA-inhibition in the dark without Fe. The greater the EDTA-inhibition, the greater the Zn-promotion, which resembles a clear-cut case of an exchange of Zn for another ion on the EDTA. It is also obvious that the combination EDTA+Zn gives a constantly higher figure than when both are absent. This indicates a slight action of Zn of its own, which occurs irregularly also in the absence of EDTA (cf. Table 7). This has warranted the inclusion of Zn in the basal solution (10^{-8} M).

The Zn-effect can be entirely explained by a small intrinsic effect on the cell multiplication and an exchange for the endogenous metal in the EDTA.

Manganese is also included as a nutrient in the basal solution. It exerts an intrinsic promotion of the cell length apparently independently of illumination (Table 8). This ought to be noticed in view of the much-discussed

Table 7. The action of Zn in combination with Fe and Mn. Darkness. Fe and Mn 10^{-6} M .

ZnSO_4 M	- Fe, - Mn		- Fe, + Mn		+ Fe, + Mn	
	cell length μ	cell number	cell length μ	cell number	cell length	cell number
0	182	194	159	231	183	296
10^{-8}	183	218	164	238	166	299
10^{-7}	162	235	162	224	156	309
10^{-6}	156	236	162	223	159	320

Table 8. *Combined growth effects of Fe^{III}, Mn and light.* All additions 10⁻⁶ M. On an average nine series of each treatment, each with six replicates.

Treatment	Light		Dark		Light effect on	
	cell length μ	cell number	cell length μ	cell number	cell length μ	cell number
-Fe -Mn	145	220	148	340	- 3	-120
-Fe +Mn	166	225	160	328	+ 6	-103
+Fe -Mn	114	198	145	333	- 31	-135
+Fe +Mn	140	207	173	329	- 33	-122
Effect of Mn						
-Fe	+ 21	+ 5	+ 12	- 12		
+Fe	+ 26	+ 9	+ 28	- 4		
Effect of Fe						
-Mn	- 31	- 22	- 3	- 7		
+Mn	- 26	- 18	+ 13	+ 1		

connexion between Mn and auxin, and also that light renders the root nearly insensitive to auxin (Burström 1960). Mn has only a slight reversing effect on the EDTA-inhibition (Table 4), which owing to a key-position of Mn in the metal sequence has been corroborated in a large number of independent experiments (cf. Table 9). It seems permissible to conclude that Mn reverses the EDTA-inhibition only slightly and hardly at all with Fe present. Its action is independent of light.

The most active metal is iron as Fe^{II} (Table 10). It significantly more than

Table 9. *Combined growth effects of Fe^{III}, Mn, and light in the presence of Na₂ EDTA 10⁻⁶ M. As Table 8.*

Treatment	Light		Dark		Light effect	
	cell length μ	cell number	cell length μ	cell number	cell length μ	cell number
-Fe -Mn	128	162	141	140	- 13	+ 22
-Fe +Mn	155	194	136	203	+ 19	- 9
+Fe -Mn	128	188	159	342	- 31	-154
+Fe +Mn	138	236	176	334	- 38	- 98
Effect of EDTA (as difference from Table 8)						
-Fe -Mn	- 17	- 58	- 7	-200		
-Fe +Mn	- 11	- 31	- 24	-125		
+Fe -Mn	+ 14	- 10	+ 14	+ 9		
+Fe +Mn	- 2	+ 29	+ 3	+ 5		

Table 10. *The reversal of the EDTA-inhibition by Fe^{II}. Basal solution + Mn, + Zn.*

Treatment	Light		Darkness	
	cell length μ	cell number	cell length μ	cell number
control	160	193	172	273
EDTA 10^{-6} M	152	192	139	150
FeCl ₂ 10^{-6} M	136	193	176	294
EDTA + FeCl ₂ 10^{-6} M	123	181	188	320

reverses the EDTA-inhibition, exhibiting a positive action of its own. This is especially obvious in the presence of EDTA. Fe^{II} induces light inhibitions in the same manner as Fe^{III}. Table 10 may serve as a type example of the iron action.

The EDTA-inhibition was also reversed by *gallium* (as Ga₂O₃·6HCl, pH 6.4—6.6) in a basal solution —Fe as follows:

addition:	none	EDTA	EDTA + Ga ^{III}		
			$3 \cdot 10^{-7}$	10^{-6}	$3 \cdot 10^{-6}$
cell number:	294	157	192	228	275.

This metal cannot be suspected to have such a physiologic effect of its own, but it promotes growth at Fe-deficiency in the presence of a chelate-forming compound. Since such compounds occur endogenously, Ga may resemble a growth promoter under other conditions as well. Analogous cases which may be explained in this way have been described in the literature (Steinberg 1941).

The complex constant of Ga^{III} is unfortunately unknown, but it may be of the same order as that of Fe^{III}.

Discussion

Uptake of EDTA

The main effect of EDTA on roots is an inhibition of the cell multiplication both in balanced and unbalanced (Burström and Tullin 1957) nutrient media on intact plants and excised roots. The roots are very sensitive to EDTA and it is possible to work with highly dilute solutions. This ought to depend upon a rapid uptake of EDTA. It is known to be taken up by plants (Weinstein *et al.* 1954). Wallace and North found equivalent uptake of Fe and EDTA, but according to Tiffin *et al.* (1960) and Brown and Tiffin (1960) EDDHA is taken up more slowly than Fe from its Fe-salt. Uptake by the roots is necessary in order to understand its ability to inhibit cell multiplication, presumably by chelating a metal inside the root, the 'endogenous' metal.

This is reversed by additions of metals, which in themselves are inactive. If metal and ligand are taken up separately the reversal must be localized inside the root and not depend upon a binding of EDTA before its entrance. We find, however, as in Figure 4 that EDTA is blocked by an equivalent amount of Fe^{III} . A separate uptake is also difficult to reconcile with the difference in activity between Fe-EDTA and an equivalent mixture of FeCl_3 and EDTA. A chelation of a metal from only the root surface can hardly explain a growth reaction, since epidermis is not necessary for growth. That the root like a chelate-former competes with external compounds for a metal has been shown by Brown *et al.* (1960).

Another possibility was that the endogenous metal was a contamination from the basal nutrient solution, all exchange in EDTA taking place in the external solution. Apart from the fact that EDTA is known to facilitate the uptake of exogenous metals, the type of water and nutrients employed permits an easy production of both iron, manganese, and boron deficiency in other materials (cf. Odhnoff 1957). The heavy metal likely to occur as contaminant would be Cu, and the EDTA would then promote growth in at least some concentrations, which has not been observed.

The endogenous metal

The *cell elongation* effect of the different metals seems to depend upon rather specific reactions of the various ions. Co and Ni act mainly as Fe-antagonists causing growth promotions only with Fe present in light, because Fe inhibits growth only under such conditions in these concentrations. Petras (1957) has explained the bacteriostatic action of Co by a blocking of Fe-enzymes. It is doubtful whether Co and Ni have intrinsic effects on the rate of elongation. The positive responses in roots are of the same magnitude as those found by Miller and others on different shoot parts. It is likewise obvious that Mn slightly promotes the rate of cell elongation both in light and darkness and that Zn has no effect.

Another picture is found with the *cell multiplication*. Hardly any effects are obtained with the metals without EDTA, but with an EDTA-addition the reversal of the inhibition may yield information about the nature of the endogenous metal involved. In the series:



Fe^{III} , Ni, Zn, and Fe^{II} strongly reverse the inhibition, Mn some, Ca and Mg not at all. Cu may be excluded owing to its obvious specific toxicity, and Co left aside owing to an inhibitory action of its own. Ga, which ought to be found to the left in the series is strongly active. De Kock (1956) obtained

a much better agreement between order of the metals and iron chlorosis in mustard leaves.

The experiments were started with the assumption that the active metal was Fe^{III} . This is obviously wrong, since it should not be strongly antagonized by Zn or Ni. On the other hand it may be difficult to distinguish between Fe^{II} and Fe^{III} since Brown and Tiffin claim that Fe^{III} is reduced to Fe^{II} on the root surface (cf. Haertl and Martell).

The tests with Fe^{II} are not conclusive either, because it could be oxidized to Fe^{III} , leaving Zn as the last active metal in the series. In favour of Zn as the endogenous metal would speak the low activity of Co and a small but significant response of an addition of only Zn. On the other hand, both Fe^{II} and Fe^{III} show the same more pronounced. The most logical conclusion would be that the active endogenous metal is Fe, perhaps together with Zn, and that *Fe is chelated from the dark-grown roots in the less stable form as Fe^{II} .*

Another type of experiments also indicate Fe as the endogenous metal. EDTA was added to a heavy-metal-free basal solution, in which the roots grew for four days; they were then transferred to and left for three days in solutions without EDTA but with applications of the metals. The idea was that EDTA should remove the endogenous metal during the initial four days. If the contaminations of the solutions are small, the roots should during the following three days suffer from deficiency in this metal, which could be cured by an addition. The number of cell divisions in darkness were:

computed	+EDTA		-EDTA for later 3 days						+complete
	first 4 days	later 3 days	none	FeIII	Ni	Co	Zn	Mn	basal solution
per 1 day	23	22	34	53	36	37	33	39	44
per 7 days	164	154	243	373	253	261	231	273	308

There are several errors in such an experiment, and they were not repeated. The added metal may not only meet a requirement but also replace a native metal in EDTA inside the root. The transport in the root of both complex and liberated metal may then be of importance for the response. The partial recovery after the fourth day without EDTA may either depend upon contaminations in the solution with the roots, or on decomposition of the complex in the root. That 60 per cent of the inhibition persists after removal of EDTA and renewal of the basal solution proves that the inhibition must be caused by EDTA taken up.

However, only Fe^{III} gives a clear response, and in spite of the errors the results indicate Fe as the endogenous metal.

The high sensitivity to the roots to low Co-additions may be due to its chemical resemblance to Fe and to competition more specific than in a chelation.

The light response

All experiments underline the close connexion between EDTA and light sensitivity of the cell multiplication. The roots become nearly insensitive to EDTA in light; the endogenous metal is then apparently not chelated. If this is iron its chelation may come to the fore in other reactions as well. One sensitive reaction indicative of the Fe-status — and probably a specific one with an excess of Mg and N — is the chlorophyll formation (cf. Oertli and Jacobson 1960). This has been determined in most of the different types of experiments recorded. It has been summarized in Figure 14, in which light response — positive for promotion, negative for inhibition — has been plotted against chlorophyll content.

The graph showing the relation between *chlorophyll and cell length* is discontinuous. The right part depicts results of the type described in Burström (1960): Fe increasing chlorophyll and light inhibition, which are both reduced by additions of Zn, Mn, Ni, and Co. The interesting feature is that the values for all these different treatments fall along one regular line, despite the fact that the actions include what was denoted as rather specific light and dark actions of Co and Ni, not to mention gibberellin. However, the curve is discontinuous when EDTA is added, starting (from the right-hand side) with negative values of light response. A possible explanation is the following one.

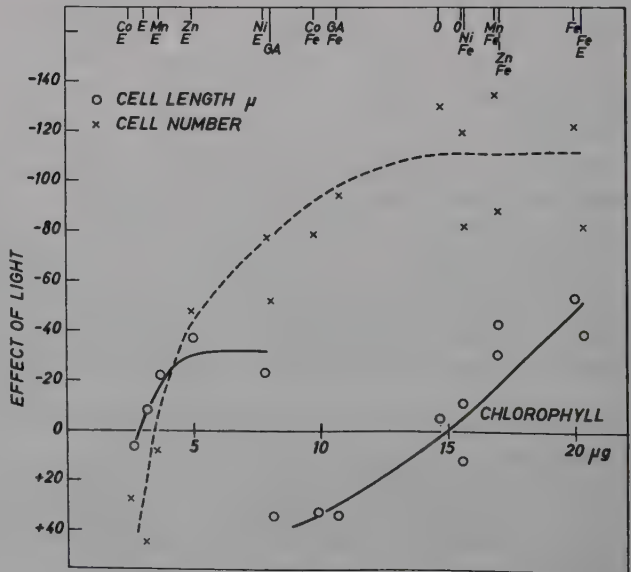


Figure 14. The relation between chlorophyll formation and growth response to light. On the ordinate effect of light on cell length and cell number (Difference between light and dark values). On the abscissa μg . chlorophyll per 100 roots in light cultures. The different additions to the solutions indicated on the top: O=no addition except basal solution, no heavy metals, E= Na_2EDTA , GA=gibberellin A3. All compounds in 10^{-6} M.

Figure 14 depicts light effects on cell length. With treatments without EDTA final cell length and rates of elongation are well correlated (cf. Figures 11, 12, also Burström 1960). According to Table 3 EDTA causes a drastic reduction in the rate of elongation in darkness, without a corresponding reduction in final cell length. Thus with EDTA light increases the rate of elongation. A graph showing the relation between *chlorophyll* and *rate of elongation* would begin with positive values (below the abscissa in Figure 14) and rise with increasing chlorophyll to inhibitions, not intersecting the abscissa until about 15 $\mu\text{g.}$ chlorophyll. The detailed shape cannot be given, because the rate of elongation was not determined for all the different treatments.

Chlorophyll and *cell multiplication* show a very regular correlation (Figure 14) regardless of kind of treatment. The light effect turns from promotion to inhibition already around 3 $\mu\text{g.}$ chlorophyll per 100 roots. Cell multiplication is about five times as sensitive as cell elongation. It was assumed that the chlorophyll formation is a measure of the active Fe in the roots. Varying external Fe-additions gives the same type of response of light sensitivity as changing the chlorophyll content (Figure 15). The intersection with the abscissa falls near 10^{-7} M for cell multiplication and at $6 \cdot 10^{-7}$ M for elongation, or the multiplication is six times more sensitive. The two graph systems of Figures 14 and 15 agree very well also quantitatively. If this is put together with the reasonable assumption that chlorophyll content reflects the iron status more than that of any other cation, it seems permissible to conclude that *iron is responsible for the light sensitivity of the roots*. Smith *et al.* found the photoinduction of Xanthium to require iron. The other ions compete with iron, and the described gibberellin effect (Burström 1960) may be of indirect importance only. It is of some interest that according to Gäumann *et al.* the fungus toxins lycomarasmin and fusarin — the latter produced together with gibberellin — may act by chelating iron.

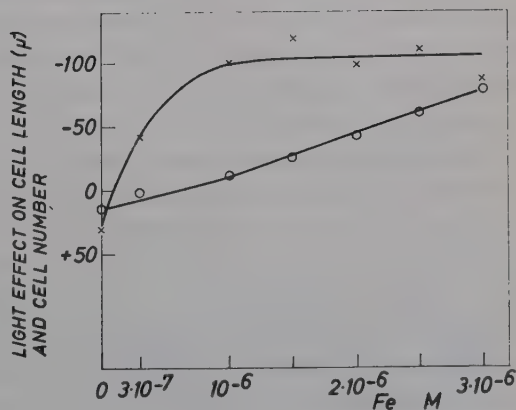


Figure 15. The influence of Fe on the light regulation of cell elongation and cell multiplication in the presence of $\text{Na}_2\text{EDTA } 10^{-6}$ M. Light effect given as difference between light and dark values. Fe added as FeCl_3 . ○ — cell length, × — cell number.

Two problems concerned with the cell multiplication are, (A) why light and dark grown roots are differently sensitive to EDTA, and (B) what Fe has to do with the light inhibition of the cell multiplication.

The first problem can be advocated in the following way. (1) The dark inhibition by EDTA is due to a chelation of Fe^{II} required as a nutrient. (2) In light EDTA is practically ineffective and cell divisions may be more rapid than in the dark with EDTA present. (3) This means either (a) that in light EDTA cannot chelate the Fe required for the divisions, or (b) that the cell divisions can do without Fe in light.

We do not know what rôle Fe has in promoting cell divisions, but it is near at hand to think of its function in respiration. This can be substituted by photosynthesis as a source of energy in the light (*e.g.* in the form of ATP, cf. Arnon 1959). The rate of photosynthesis in the greening roots has been determined (by A. Fadeel) and it does by far not reach the compensation level; whether the Fe requirement for photosynthesis is less than in respiration is also doubtful. The explanation (3 b) does not seem to be very likely, leaving (3 a) as the most probable answer.

Question (B) implies that (4) Fe functions in a system which inhibits cell divisions in light, and (5) this Fe cannot be removed by EDTA.

The following very tentative picture would explain the available results. In light Fe, which is indispensable for cell divisions, occurs predominately in such a form or in such a binding that it cannot be chelated by EDTA. In this firmly bound form it also takes part in a system causing an inhibition of cell divisions and is linked up in the formation of chlorophyll or the chlorophyll function. In the dark Fe is less firmly bound and easily removed.

Haertl and Martell (1956) mention that $\text{Fe}^{\text{III}}\text{EDTA}$ is light sensitive, and in sunlight its iron is reduced to Fe^{II} . If this holds true under our light regime it would accentuate the importance of Fe^{II} as the active metal. However, it could under no circumstances explain the light sensitivity of our system, because the relations between light, iron, and growth are the same with EDTA absent and only Fe^{II} supplied, according to Table 2. The light response cannot depend upon the properties of the EDTA. This only serves as a vehicle for metals.

It is tempting to consider the reversible system $\text{Fe}^{\text{III}} \rightleftharpoons \text{Fe}^{\text{II}}$ and the very different complex constants of tri- and di-valent iron. Fe^{III} chelates are much more stable. With EDTA Martell and Calvin give the $\log K$ values for Fe^{II} and Fe^{III} as 14 and 25 respectively; theoretically also native chelates should be more stable with Fe^{III} than with Fe^{II} . In light Fe could be oxidized to Fe^{III} , which Lundegårdh (1954) was the first actually to demonstrate in cytochrome *f*, and which according to Arnon (1959) enters into the mechanism of photophosphorylation. The correlation between chlorophyll con-

tent and light-sensitive growth may be mediated by energy transformations. Fe could also be reduced in the light and thus the chelation by EDTA weakened relative to that by the native ligand. Other possibilities are that if the stable light-form of Fe is involved in chlorophyll formation, the pigments themselves or products of photosynthesis may be directly or indirectly responsible for the light-growth reactions. Either mechanism will place iron in a key-position in the light-sensitive growth system.

Summary

A study has been made of the influence of EDTA and light on the growth of excised wheat roots, and the actions of Ga, Fe^{III}, Cu, Ni, Co, Zn, Fe^{II}, and Mn on the growth. This has been computed as rate of cell divisions in the meristem, rate of cell elongation, and final cell length attained.

EDTA inhibits strongly the cell divisions in the dark but not in the light. This is reversed by Ga, Fe^{III}, Ni, Zn, and Fe^{II}, less by Co, slightly by Mn, not by Ca or Mg.

Light inhibits cell divisions, making the roots practically insensitive to both EDTA and metal additions. The light sensitivity closely parallels the formation of chlorophyll, which is supposed to depend upon the Fe-status. All results indicate that EDTA changes growth by chelating from the root foremost Fe^{II}. It is assumed that Fe occupies a key-position in the growth and light-growth regulation. The different sensitivity of the roots to EDTA in light and darkness has been discussed, also the correlation between growth reactions and chlorophyll formation. A tentative explanation is that in light Fe occurs in a stable chelate — perhaps as Fe^{III} —, which is involved in both growth and the chlorophyll system.

It has been confirmed that Fe is also necessary for the light inhibition of cell elongation. The actions of other cations, notably Co and Ni, can mainly be explained by an exchange for Fe.

Some specific metal actions were observed: slight promotions of cell multiplication by Zn and of cell elongation by Mn, a strong toxicity of Cu, a lower toxicity of Ni in light only, and an inhibition of cell divisions by low concentrations of Co.

A probably partly autonomous spiral growth of the excised roots has been observed.

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Growth Chamber for Labelling Plant Material Uniformly with Radiocarbon

By

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Radiocarbon (C^{14}) is a very useful tool in the study of the transformation of soil organic matter. A full utilization of the isotope for this purpose requires that uniformly labelled plant material can be metabolized in the soil.

A uniformly labelling of plant material with radiocarbon can be achieved by growing the entire plant during its whole growing period in an atmosphere containing carbon dioxide with a constant specific activity. The only way to procure such conditions is to grow the plant in a closed system. This requires, however, an accurate and reliable control of the plant environmental factors within the unit. Growth chambers fulfilling these requirements have been in use for some time now, e.g. at the Argonne National Laboratories, U.S.A. (Scully *et al.* 1956) and at the Institute for Agricultural Chemistry, University of Bonn, Germany (Sauerbeck 1960).

The growth chamber described in this paper has been constructed mainly for the production of plant material uniformly labelled with radiocarbon. Due to the accurate and reliable control of the environmental factors the chamber might also be used for other plant physiological experiments.

The Growth Chamber

The growth chamber is of a permanently fixed type and made of stainless steel. It has the shape of a box, its base being 1 by 1.5 m and the height 2 m. The back is fitted with an airtight door, the front is provided with an

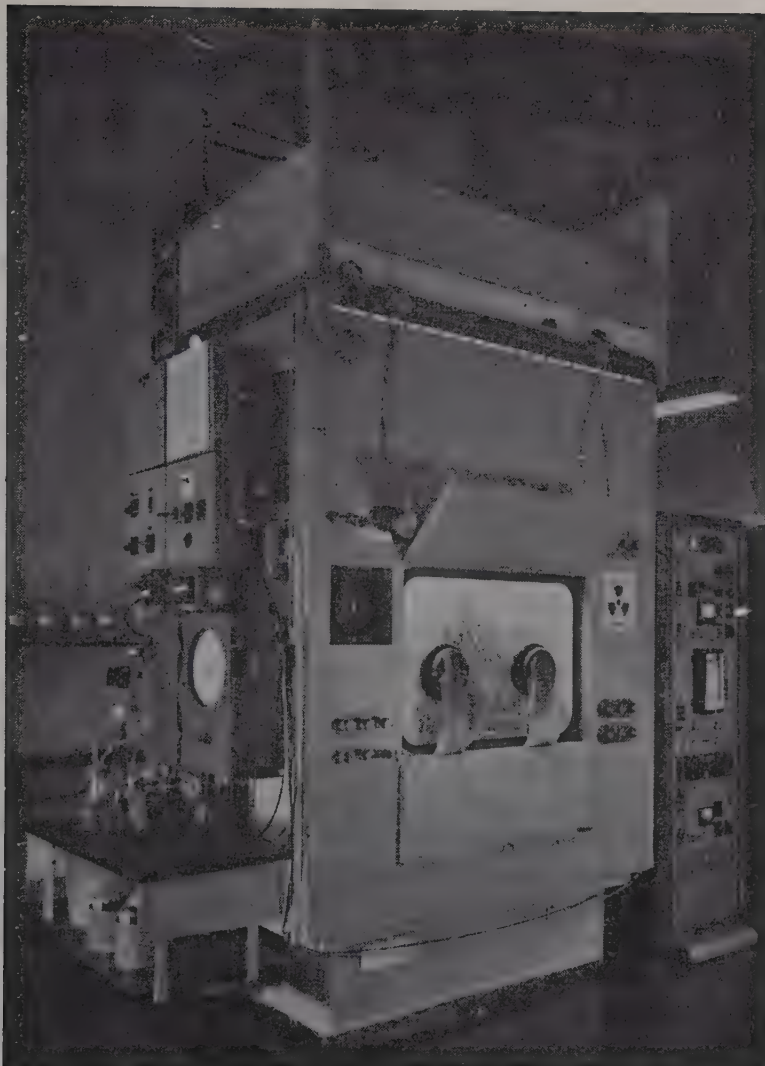


Figure 1. *The growth chamber. To the right the electronic equipment for counting and recording the C¹⁴ activity. To the left the equipment for control of the air conditioning and the CO₂ analyser and recorder.*

observation window of plexiglass having 3 port holes, 2 of which can be fitted with gloves, the third one positioned to allow the removal of material from the chamber whilst under operation (Figure 1). Sealed service inlets and outlets are fitted to the front and back of the chamber. The top of the chamber is a glass pane above which the light sources are placed.

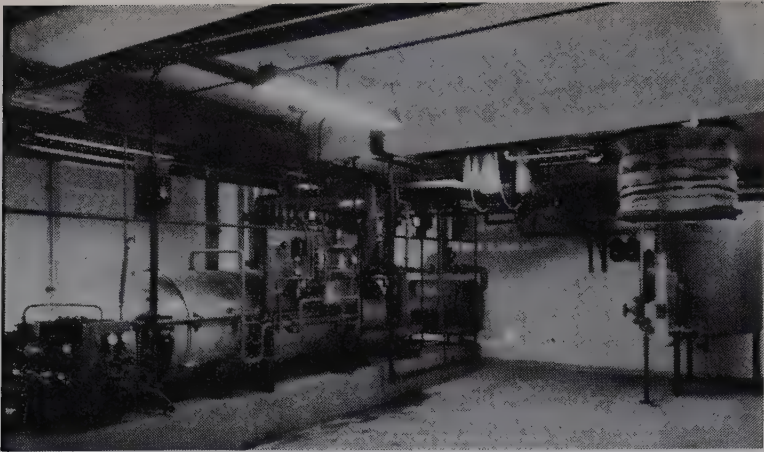


Figure 2. The basement below the growth chamber. To the left the machinery performing the air conditioning. To the right the expansible bag for regulation of the air pressure in the chamber and the tank for the nutrient solution.

The inorganic nutrients are provided from a solution periodically pumped up from a reservoir tank lying in the basement of the building. The lower portion of the chamber acts as a bassin for this solution. Baskets containing the rooting medium (perlite) for the plants are placed in this part of the chamber and are periodically flooded by the nutrient solution.

The temperature and humidity of the air in the chamber can be controlled automatically by means of an internal air circulation system, containing cooling and heating surfaces and a set of radiators for flow of cold or hot water. The compressor and accessory machinery used for the air conditioning system are placed in the basement (Figure 2).

The carbon dioxide concentration and the carbon-14 activity of the air in the chamber is measured, recorded and controlled automatically.

Control of the Plant Environment

Temperature and humidity of the air. The evaporation of water from the plants and from the surface of the nutrient solution is so large that a regulation of the humidity can be performed by means of a condensation system. A diagram of the arrangement for an automatic regulation of air temperature and humidity is shown in Figure 3. An exhaust outlet (B) for the air to the internal circulation system (E) is placed just above the surface of the nutrient solution. In the circulation system the air passes the cooling surface (G) whose temperature is controlled by the hygrostate (J) and the valve (O). A certain part of the humidity condenses there and is returned to the tank containing the nutrient solution. The air is passed over

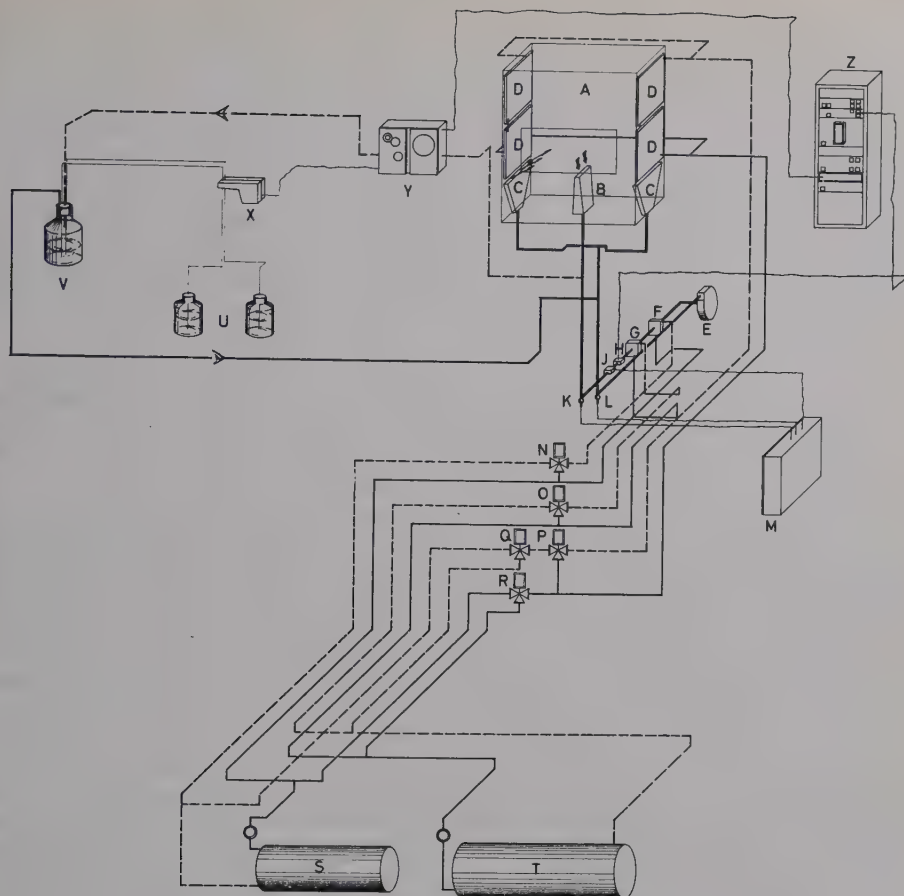


Figure 3. Diagram showing the air conditioning system and development of C¹⁴O₂. A full line indicating inlet, a broken line outlet of water. A. The growth chamber. B. Exhaust outlet of the internal air circulation system. C. Nozzles for inlet of air from the circulation system. D. Radiators for flow of cold or hot water. E. Pump for air circulation. F. Heating surface. G. Cooling surface. H. Detector for the C¹⁴ recording. J. Hygrostate which regulates valve O for flow of cold water to the cooling surface. K. Thermostate which regulates the valves P, Q, R for flow of cold or hot water through the radiators D. L. Thermostate which regulates valve N for flow of hot water to the heating surface. M. Panel for electronic equipment performing the regulations. N, O, P, Q, R. Valves regulating the flow of cold or hot water through radiators, heating and cooling surfaces. S. Container for hot water. T. Container for cold water. U. Containers for potassium carbonate solution and lactic acid. V. Vessel for producing CO₂. X. Pumps drawing potassium carbonate solution and lactic acid from U to V. Y. CO₂ analyzer which records the CO₂ content of the air in the chamber and regulates the pumps X. Z. Electronic equipment for counting and recording the C¹⁴ activity of the air in the chamber and for regulating the pumps X.

the heating surface (*F*) whose temperature is controlled by the thermostat (*L*) and the valve (*N*) so that the difference in temperature of the inlet and outlet air does not exceed 8°C. The air is blown into the chamber again through a number of nozzles (*C*) placed along the lower edge of the radiators (*D*) at both sides of the chamber. In case the cooling and heating surfaces fail to be able to regulate the desired temperature a set of 3 valves (*P*, *Q*, *R*) are adjusted by the thermostat (*K*) to allow a flow of hot or cold water through the radiators (*D*).

Independently of the above mentioned apparatus a continuously measuring and recording of the dew-point temperature and the air temperature is performed automatically. The relative humidity in the chamber can be regulated within the range of 50–80 % with an accuracy of ± 3 %, and the air temperature within the range of 10–35°C with an accuracy of $\pm 0.5^\circ$.

The carbon dioxide concentration of the air. The carbon dioxide of the air in the chamber originates from a solution of potassium carbonate with a known content of C^{14} . The potassium carbonate solution and a lactic acid solution are drawn from their respective containers (*U*) by means of a two headed pump (*X*) into the vessel (*V*) to produce carbon dioxide. A small current of air is taken from the outlet vent of the circulation system and let through an infra-red carbon dioxide analyzer (*Y*) which regulates the pump so that the carbon dioxide concentration of the air remains constant within certain limits. The air having passed the carbon dioxide analyzer is let into the production vessel (*V*) where it is mixed with newly produced carbon dioxide and fed back to the chamber via the inlet vent.

The concentration of the carbon dioxide of the air is recorded by the analyzer, and can be regulated within three different ranges, i.e., 0–0.1, 0–0.3 and 0–1.0 % CO_2 . By choosing a proper concentration of the carbonate solution and by having a slow rate of pumping a high accuracy can be obtained, e.g., a certain concentration within the range 0–0.1 % CO_2 can be obtained with an accuracy of ± 0.005 %.

As the carbon dioxide concentration of the air increases during the dark period due to the respiration of the plants a carbon dioxide absorber can be inserted and coupled to the analyzer to keep the carbon dioxide concentration constant independently of the illumination.

The air pressure, which increases gradually due to photosynthesis can be kept constant in relation to the atmosphere by means of an expansible bag.

The radiocarbon concentration of the air. The C^{14} concentration of the air is measured and recorded automatically. A proportional-flow counter (*H*) with a very thin window is placed in the outlet vent (Figure 3). The counting rate which is an arbitrary expression for the activity of the air is recorded by a count ratemeter connected to a recorder (*Z*). This system can have the same function as the carbon dioxide analyzer so that it is possible by means of a change over switch to decide which one of the two systems has to perform the control of the carbon dioxide production. The two systems work independently but are for safety reasons connected so that in case of a functional disturbance of one of the systems the other is able to stop the carbon dioxide production when a certain maximum concentration level has been exceeded.

Inorganic nutrients. The baskets containing the rooting medium (perlite) are made from galvanized, perforated sheet iron coated with asphalt varnish. The baskets are suspended in the bottom part of the chamber in such a way that the upper surface of the rooting medium is about 5 cm above the maximum liquid level which is kept by a reflux tube connected with the reservoir tank in the basement. The

nutrient solution (Hewitt 1952, p. 189—190) is automatically pumped from the tank to the chamber 1, 2 or 3 times a day. When the pump stops the nutrient solution returns to the tank which has a capacity of 800 l. Addition of nutrients and removal of samples for analysis can be done through a stopcock on the tank.

Light. The lighting in the chamber is entirely artificial. The lighting panel is separated from the chamber by a glass pane and consists presently of 20 fluorescent tubes of 80 watt each and 5 incandescent lamps (reflector type) of 100 watt each. The lamps are cooled by a current of air which is constantly drawn over the lighting panel. The maximum light intensity is about 10,000 Lux at the level of the rooting medium. The alternation of light and dark periods is effected by an automatic control via a time-clock. As the light is switched off a drop in the air temperature of up to 15°C can be obtained by an automatic insertion of a resistance in the system for the temperature control.

Summary

An automatically regulated growth chamber is described. It can be used to grow plants over their entire growing period in an atmosphere of constant radiocarbon dioxide concentration. As the plant environment inside the chamber can be accurately and constantly controlled, the unit may be used as a radioisotopic biosynthesis facility or as a modified phytotron in plant physiological studies.

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On the Formation of Protochlorophyll in Normal Green Wheat Leaves of Varying Age

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Introduction

The chlorophyll *a* immediately detectable in irradiated leaves of seedlings which have previously been kept in darkness, derives from protochlorophyll (Koski *et al.* 1951, Smith 1960, Virgin 1955 a). This pigment is formed slowly in darkness and the rate of its formation is the limiting factor for the subsequent formation of chlorophyll *a* in light. This applies at least to the first stages of the greening process (Virgin 1955 b). Due to the rapid transformation of protochlorophyll into chlorophyll *a* in light (Koski *et al.* 1951, Smith and Benitez 1954), it is difficult to trace protochlorophyll, active at the transformation, in leaves which have been irradiated for a longer period of time. The small amount of protochlorophyll-like pigment which can sometimes be found in leaves kept in light seems to be another form of the substance, inactive at the transformation, but with the same absorption and fluorescence properties *in vitro* (Shibata 1957, Virgin 1960).

Protochlorophyll in normal, green leaves has been reported by Smith and Koski (1948), Litvin *et al.* (1959) and Virgin (1960). Doubt has been expressed that protochlorophyll is the precursor to chlorophyll *a* throughout its continuous accumulation (Virgin 1955 b), cf. Smith (1960). Through the use of radioisotopes Litvin *et al.* (1959) and Duranton *et al.* (1958) have presented evidence which point to protochlorophyll being the precursor also to the chlorophyll *a* formed during later stages of the greening. If all chlorophyll *a* which is present in leaves has come from protochlorophyll, a study of the rate of formation of this substance at different developmental stages of the

leaves would give a measure of the turnover of chlorophyll *a* during the development of the chlorophyll forming organ.

In the present study the formation of protochlorophyll in the primary green leaves of wheat, placed in darkness at different stages of their growth, has been measured. It is shown that protochlorophyll is formed only as long as the leaf is still growing. When all cells are fully stretched, and the final size is reached, also the formation of protochlorophyll ceases.

Material and Method

Primary leaves of wheat (Weibull's Eroica II) were used for the experiments. The plants were cultivated in nutrient solution on screens of stainless steel in accordance with Virgin (1958). The cultivation took place in a thermostatically regulated room illuminated with fluorescent tubes (Philips TL 65W/29). The photoperiod was 18 hours and the intensity at the cultivation level 4000 lux. The temperature was kept at 12°C during night and at 15°C during daytime. From the cultures leaves were taken every day at the same time and placed in glass tubes, wrapped in tinfoil. They were then kept in darkness at 22°C for 5 hours, whereafter they were frozen at -26°C. The lowering of the temperature will immediately stop the further formation of protochlorophyll, and the actual contents of pigments will become preserved. For the pigment analysis the method earlier described was followed (Virgin 1960). The leaves were ground with sand and acetone under dim green light. The pigments were then separated by ascending paper chromatography according to Virgin (1960) using a mixture of acetone and petroleumether in the ratio 15 : 85 by volume as developer (in the paper by Virgin 1960, the proportions were wrongly given as 85 : 15). With this developer all the active protochlorophyll remains within the first 16 millimeters of the paper. The quantitative determination of the pigments were done spectrofluorimetrically. The inactive part of the protochlorophyll (Virgin 1960) has not been included in the values for protochlorophyll.

Experimental

When normal green irradiated leaves of wheat have been kept in darkness for some hours small amounts of protochlorophyll can be traced (Virgin 1960). The formation of the protochlorophyll goes on until a certain concentration level is reached. From Figure 1 it can be seen that the rate of the formation is strongly affected by temperature, like the conditions in etiolated leaves of barley (Virgin 1955 b). Probably also the final concentration level varies with temperature. From the experiments on the effect of light on the lag phase of protochlorophyll formation (Withrow *et al.* 1956, Wolff *et al.* 1957, Virgin 1957, 1958) the conclusion can be drawn that the initial rate of protochlorophyll formation in darkness in a normal green leaf, pre-

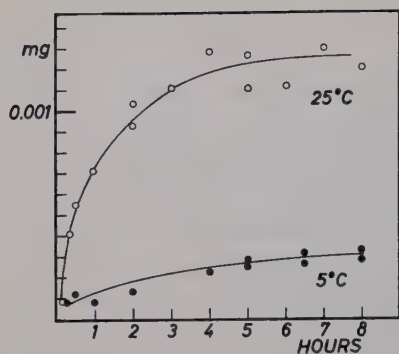
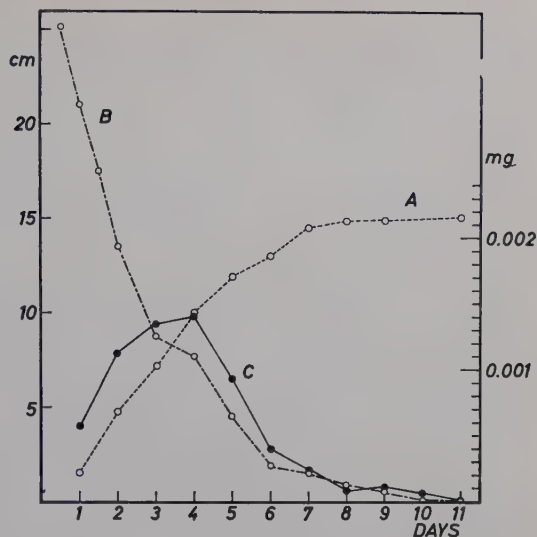


Figure 1. *Protochlorophyll formation in darkness in normal green leaves of wheat.* Abscissa: Time in hours. Ordinate: Protochlorophyll in milligrams per gram of fresh weight. Age of leaf four days.

viously kept in light, must be greater than that in an etiolated leaf which has been shortly irradiated and then placed in darkness. In the normal green leaf, the light, administered continuously prior to the dark period during which the protochlorophyll formation is followed, has been acting for such a long period of time that its accelerating effect on the subsequent protochlorophyll formation is fully reached. In the etiolated leaf a period of at least four to six hours must elapse after a light impulse in order for this effect to become maximal (Virgin 1957). It is difficult, however, experimentally to show this difference as the final level of protochlorophyll which will be obtained in a green leaf, kept in darkness during 5—8 hours, is always considerably lower than that reached in a previously dark grown leaf without any chlorophyll. It seems probable that the presence of chlorophyll in the leaves depresses the protochlorophyll formation.

The amount of protochlorophyll formed in the leaves, kept in darkness for a certain period of time, is dependent on their developmental stage. In Figure 2, curve B, it can be seen that the concentration of protochlorophyll after five hours in darkness, calculated on fresh weight basis, is great when the leaves are at the beginning of the period of elongation, but decreases rapidly as the stretching of the cells take place. When the leaves have reached their final length after about ten days under the prevailing conditions, no measurable amounts of protochlorophyll are formed during a period of five hours in darkness. From this the conclusion can be drawn that protochlorophyll is formed in the leaves only during the time of development when the total amount of chlorophyll is steadily increasing. During this period of development the concentration of chlorophyll *a* is increasing for the first four days, and then remains constant at about 0.9 mg. per gram fresh weight. This implies that during this later phase of the development

Figure 2. *Protochlorophyll formation during five hours in darkness in normal, green leaves of wheat of varying age. Temp. 22°C. A. Length of the leaves in centimeters (left ordinate). B. Protochlorophyll in milligrams per gram of fresh weight (right ordinate). C. Protochlorophyll in milligrams per ten leaves (right ordinate). Every point mean of three determinations.*



the absolute increase in chlorophyll *a* per cell unit keeps step with the stretching of the cells. (Cf. Wiećkowski 1960.)

The amount of chloroplasts per cell unit is supposed to be more or less constant when the cells are fully stretched (cf. Küster 1951), and the chloroplasts do not divide after the end of the cell division. This means that different values for the protochlorophyll content would be obtained when calculated on weight basis as compared to calculation on cell basis (pro leaf unit). This would be the case at least when the leaves are young, provided that the increase in weight only consists of a stretching of the cells. In grass leaves, however, a long-lasting meristematic activity persists at the base of the leaf blade (cf. Esau 1953) resulting in an increase in the total amount of cells as the leaf is increasing its surface. In spite of this, a calculation per leaf unit will give a certain information of the pigment formation per cell. The protochlorophyll content in darkness after five hours, calculated per unit leaf is seen in Figure 2, curve C. One can notice a steadily decreasing amount of newly formed protochlorophyll during the dark period as the leaves get older, starting after the fourth day. It is of interest to notice that in the earlier stages of development the chlorophyll formation mechanism has not yet developed its full capacity. The maximum rate of protochlorophyll formation is obtained when the leaves have reached an age of 3–4 days, whereafter the rate steadily decreases with the ageing of the leaf. This first increase in the rate of protochlorophyll formation may also partly depend on the abovementioned continued meristematic activity. It is to be

noticed that the maximum in the rate of protochlorophyll formation coincides with the time when the concentration of chlorophyll *a* in the leaves becomes constant.

Discussion

The turnover of chlorophyll

It is generally believed that the more or less constant level of chlorophyll pigments in a normal fully developed green leaf is the result of a continuous synthesis and decomposition of pigments (cf. Egle 1960). A very stable equilibrium is thus supposed to exist between newly formed and decomposed chlorophyll, at least during longer periods of time.

Particularly the relationship between the chlorophyll destruction in darkness and the new formation in light has come in the center of this problem. If a destruction takes place in darkness, the question is whether this goes on also in light, and whether under these conditions it is counteracted by a simultaneously occurring new formation of pigment. That a destruction of chlorophyll in darkness can take place also during such a short period of time as one night has been shown by several authors. But as the conditions differ greatly due to the developmental stage of the leaves and to specific characters, many conflicting reports are found in the literature (cf. Wiećkowski 1960).

In etiolated, shortly irradiated leaves of corn seedlings, a rather rapid destruction of the chlorophyll is reported to take place when the leaves are again placed in darkness at 27°C (Frank and Kenney 1955). The rate of this destruction seems to depend upon the nutrition level, as an addition of sucrose to the nutrient solution strongly decreases the rate of decomposition. Applied to the nutrition medium sugar has even a strong positive effect on the formation of chlorophyll (Wolff and Price 1960). In normal green leaves which during daytime have photosynthesized and thus have a high nutrition level the breakdown of the chlorophyll pigments during the following night can therefore be supposed to be much lower than in plants deprived of sugars. The rate of decomposition of chlorophyll in darkness also seems to vary considerably from one plant species to another. In fact no chlorophyll destruction could be established in etiolated wheat plants, shortly irradiated, and then afterwards placed in darkness for five hours (Virgin 1958). When a destruction is present in darkness, it becomes more evident the older the leaves (Wiećkowski 1960).

The degree of the turnover of chlorophyll has been the subject for discussions for a long time and there are conflicting reports in the literature. The

values obtained are often based on studies on daily changes in chlorophyll content. Such daily changes are particularly predominant in young leaves which are still growing, but become smaller or disappear when the leaves are fully developed (Mitrakos 1960, Wiećkowski 1960). Daily changes in fully developed leaves have also been reported in some instances but have not been confirmed by other workers. According to Seybold and Falk (1959) there are no indications of shortlasting changes in the concentration level of chlorophyll *a* and *b* in a normal fully developed green leaf. In a critical study of the available data concerning the matter, these authors have reached the conclusion that the hitherto reported changes can be brought back to imperfections of the method used for the determinations, and to the fact that the measurements were made on not fully developed leaves. Studies on the incorporation of radioisotopes also give conflicting results. While some Russian authors (see Bavrina 1959) report a continuous exchange of radioactive material, indicating a continuous synthesis and decomposition of pigments, other authors (Perkins and Roberts 1960) have not been able to confirm such a turnover in fully mature leaves. In still growing leaves, however, incorporation of radioactive material in the pigments take place.

As far as leaves of wheat are concerned, Williams (1960) found that primary leaves of wheat seedlings have completed their growth after about 8 days. And the same is valid for all consecutive leaves, with an average time of about 8—10 days for the complete development. Studying the incorporation of ^{14}C into the dihydroporphyrins of 5—13-day-old primary leaves of Kharkow 22 M.C. wheat, Perkins and Roberts (1960) found that the 13-day-old primary leaves did not incorporate any ^{14}C , while the 5-day-old leaf did. From this the conclusion was drawn that the mature leaves do not synthesize any measurable amounts of chlorophyll.

Comparing the experimental results reported in the present paper with the above-mentioned values for the turnover of chlorophyll pigments in wheat leaves, measured by means of radioisotopes, one finds a good agreement. In Figure 2 it was shown that new formation of protochlorophyll which is rapid when the leaves are young, decreases rapidly during ageing. After about 10 days when the final length is reached, very little, if any, protochlorophyll can be found in leaves which have been in darkness for five hours. These results together with the earlier found fact that this protochlorophyll will immediately be transformed to chlorophyll *a* at irradiation (Virgin 1960) give further support to the assumption that, firstly, the protochlorophyll also during the later stages of the process of greening is the precursor to the chlorophyll *a* and, secondly, the turnover of chlorophyll *a* in fully green and mature leaves is very low or is lacking.

The fact that the formation of chlorophyll ceases when the leaves are

full-grown does not necessarily mean that the growth of the leaf blade and the synthesis of chlorophyll are dependent processes (cf. Klein *et al.* 1957). In plants, transferred to darkness, the chlorophyll formation is immediately stopped, whereas the growth of the leaves continues for some time. In studying bean leaves, Wiećkowski (1959) found a high rate of new formation of chlorophyll in plants kept in darkness for a few days and then exposed to light. It is to be noticed, however, that such a new formation of chlorophyll during the following light period takes place only when the leaves have continued to grow during the dark period, when thus a "dilution" of the pigments have occurred (Wiećkowski 1960). If they are fully developed prior to the transfer to darkness, firstly, they can stand the darkness for only a few days, and secondly, no further chlorophyll will be formed in light.

Daily changes in chlorophyll formation

From the findings in this paper concerning protochlorophyll formation in darkness in growing leaves, one can estimate the course of the changes in the synthesis of chlorophyll *a* in growing leaves of a plant which is exposed to the daily changes in darkness and light. During the light period of the day practically no protochlorophyll will be present in the leaf as the photochemical reaction protochlorophyll \rightarrow chlorophyll *a* is extremely rapid, (Koski *et al.* 1951, Smith and Benitez 1954). New formation of chlorophyll *a* takes place continuously and the rate of this formation is limited by the rate of protochlorophyll formation. From the experimental evidence one can draw the conclusion that at decreasing light intensities (nightfall) the balance between protochlorophyll formed and protochlorophyll transformed into chlorophyll *a* will become shifted more and more towards the left the weaker the light intensity. At dawn, the opposite changes take place. In complete darkness no chlorophyll *a* is formed at all and in the middle of the night the maximum value for protochlorophyll accumulation will be reached. Depending upon the length of the dark period the concentration level will vary, but after a certain time in darkness the protochlorophyll formation will cease. Whether the maximum value for the protochlorophyll level will be reached depends on the length of the night. In short nights this will never happen and new protochlorophyll formation takes place continuously. If the nights are long enough, the maximum level will be reached and the formation of protochlorophyll will stop for a while. This fact may have bearings on the photoperiodic response of different plants. One would expect this as also the length of day has an influence on the chlorophyll content (Sironval 1959, Friend 1961).

From the afore-mentioned studies on light effects on the lag phase in

protochlorophyll formation (Withrow *et al.* 1956, Wolff *et al.* 1957, Virgin 1958) it can be concluded that the rate of formation of chlorophyll *a* after some hours of light should be a little higher than at dawn.

The daily changes which have been outlined here will continue as long as the leaves are still growing. They will cease gradually as the stretching of the cells decreases and when fully mature, the changes in the chlorophyll *a* content are probably very small.

Summary

When placed in darkness, normal still growing green leaves produce small amounts of protochlorophyll. The rate of this formation is high at the beginning of the dark period and decreases until a certain more or less constant value for the concentration is reached after about 6—8 hours.

The protochlorophyll formed in green leaves of wheat of varying age after a stay in darkness for 5 hours has been measured. It is shown that the concentration level after this time in darkness is steadily decreasing with the age of the leaf when calculated on a weight basis. When calculated on a leaf unit basis a maximum value is obtained when the leaf is 3—4 days old, whereafter the protochlorophyll decreases with age. After about ten days no more protochlorophyll is formed.

The daily changes in chlorophyll content in leaves under natural conditions are discussed on the basis of the values for protochlorophyll formation in darkness.

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The Action of Gibberellic Acid in the Slit Pea Stem Curvature Test

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I. Introduction

According to Sumiki (1952), Kato (1953), and Hayashi and Murakami (1958), gibberellin is without action in the slit pea test. This is somewhat surprising, since it is well known that gibberellin exerts a stimulating effect on growth of pea stem sections (see *e.g.* Brian, Hemming and Radley 1955, Hayashi and Murakami 1958, Kato 1958, Purves and Hillman 1958). True, this effect is very slight as compared to the dramatic effect of gibberellin on growth of intact pea plants (Brian and Hemming 1955), but it should nevertheless be of a sufficient magnitude to give a measurable response in the slit pea test. It was therefore thought to be worth while to reinvestigate the action of gibberellin in this test.

II. Materials and Methods

Seeds of dwarf peas (Kelvedon Wonder) were sterilized for 15 minutes in a 1 % solution of calcium hypochlorite. They were next rinsed for 1 hour in running water and were soaked for 4 additional hours in tap water. The imbibed seeds were sown in moist perlite (crushed pumice) in earthenware dishes, and were then placed in the darkroom at a temperature of 23—24°C. As soon as the seedlings were visible, they were daily exposed for 30 minutes to red light from a photographic darkroom lamp. Otherwise, only green light was used during work in the darkroom. The light source consisted of a green gelatine filter (Ilford Spectrum Filter No. 604) in combination with a green fluorescent tube. This filter has a maximum transmission at 524 mμ, and its transmission in the visible region is negligible above 550 mμ and

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below 500 m μ . Above 760 m μ there is some transmission. But radiation above 760 m μ seems to affect photomorphogenetic reactions only slightly (see *e.g.* Mohr 1960). Furthermore, the usual types of fluorescent tubes produce but small amounts of light in the infrared region. This type of green light should therefore be well suited for darkroom work with higher plants (*cf.* Withrow and Price 1957).

For these experiments, the third internodes of the seedlings were used. As a rule, the test plants were of a suitable size 8 days after sowing, the third internode then having a length of about 5 cm.

Sections of 4 cm. length were cut from the third internodes, just below the point where the internodes begin to attain a uniform thickness, the apical ends of the sections thus corresponding in position to the apical ends of the S 4 sections used by Purves and Hillman (1958).

The sections were placed in a groove in a cork and by means of a razor blade slit medially 3.5 cm. downwards from their apical ends. The slit sections were washed for 1 hour in distilled water and were then transferred to the test solutions. Test solutions were buffered with Sørensen's phosphate buffer (0.003 M, pH 7.0). The tests were carried out in 8 cm. petri dishes. Twenty ml. of test solution and 3 sections were used per dish.

After 20 hours the sections were removed from the test solutions. Adhering moisture was wiped off with filter paper. The sections were then dipped in Indian ink and carefully pressed against a sheet of paper. On the prints thus obtained, curvatures were measured according to the inflection point method (Went and Thimann 1937).

A factorial experimental design was used. Six levels of gibberellic acid (GA) and six levels of indole-3-acetic acid (IAA) were tested in all possible combinations, giving a total of 36 different treatments. Concentrations tested were, of GA 0, 0.001, 0.01, 0.1, 1 and 10 mg/l, and of IAA 0, 0.01, 0.1, 1, 10, and 100 mg/l. As a rule, 6 sections were used in each treatment, and the whole program was run through twice, giving a total of 12 section, *i.e.* 24 curvatures, per treatment. Simultaneously with each combination experiment a control with IAA only was always run, equal concentrations of IAA being applied in control and combination experiments.

GA and IAA were dissolved in 0.1 M Na₂CO₃, the excess of which was neutralized with 0.1 N HCl (Larsen 1955, p. 590). Test solutions were always prepared immediately before use.

III. Results

The results are presented in Table 1. In this set of data, the group standard deviations are strongly dependent on group means. It was therefore deemed advisable to make a logarithmic transformation before carrying out the analysis of variance. The results of the analysis of variance are given in Table 2. There seems to be very little doubt that GA is effective in the slit pea test, its principal action being to increase curvatures caused by simultaneously applied IAA.

Obviously, the GA response (increase in curvature caused by GA) at a certain level of GA increases with increasing concentrations of IAA. But there

Table 1. *Slit pea stem curvature test*. Mean curvatures in degrees produced by different combinations of gibberellic acid (GA) and indole-3-acetic acid (IAA).

Concentration of IAA, mg/l	Concentration of GA, mg/l					
	0	0.001	0.01	0.1	1	10
0	0.3	1.9	3.5	0.4	0.9	0.0
0.01	9.1	11.9	8.6	20.5	17.0	14.7
0.1	93.3	135.2	110.5	118.3	98.5	129.4
1	208.0	219.0	250.3	303.2	272.8	252.9
10	231.7	213.1	221.3	259.0	273.6	303.5
100	281.8	248.3	284.6	232.7	371.0	343.0

is also evidence that too high concentrations of IAA have a depressing action on GA responses produced by GA concentrations up to 0.1 mg/l. In other words, low levels of GA (0.01 and 0.1 mg/l) give a maximal response at a relatively low level of IAA (0.1 and 1 mg/l). High levels of GA (1 and 10 mg/l) require high levels of IAA (10 and 100 mg/l) for a maximal response.

The IAA effect needs no comment. As regards the GA effect, it should be mentioned that even pure GA solutions seem to be effective. The response is very slight, however, and rather irregular. The effect of 0.001 mg/l GA does not seem to be statistically significant.

Clearly, if the relation between GA response and IAA concentration is different at different concentrations of GA, as suggested above, there must be a statistical interaction between high and low levels of GA. To test this possibility, a new analysis of variance was made, omitting the 0 GA level. The interaction term proved to be statistically significant at the 0.1 % level.

However, this result might also be explained by the null hypothesis that low levels of GA (0.001, 0.01 and 0.1 mg/l) are without effect in the slit pea test. This was disproved by a third analysis of variance. This time, the two highest levels of GA (1 and 10 mg/l) were omitted, and the 0 GA level was again included. The interaction was statistically significant at the 1 % level.

Table 2. *Slit pea stem curvature test*. Analysis of variance of transformed data.Transformation: $x' = \log(100 + x)$.

Source of variation	Sum of squares	Degrees of freedom	Mean square
Interaction	0.77608	25	0.0310*
GA main effect ..	0.39157	5	
IAA main effect ..	50.74184	5	
Residual	4.37022	953	0.00459

* Interaction statistically significant at the 0.1 % level.

Table 3. *Slit pea stem curvature test*. Mean curvatures in degrees produced by different levels of gibberellic acid (GA) in combination with different concentrations of IAA. Pooled data. Further explanation in text.

Concentration of IAA, mg/l	GA level		
	0 and 0.001	0.01 and 0.1	1 and 10
0	1.1	2.0	0.5
0.01	10.5	14.6	15.9
0.1	114.3	114.4	114.0
1	213.5	276.8	262.9
10	222.4	240.2	288.9
100	265.1	258.7	357.0

Furthermore, no interaction seems to exist between the two highest GA levels. These two levels may therefore be pooled. Similarly, it is permissible to pool 0 and 0.001 mg/l GA, and also 0.01 and 0.1 mg/l GA. Table 1 may thus be simplified. The figures resulting from this simplification are given in Table 3.

An analysis of control experiments with pure IAA solutions revealed some variation between occasions. The deviations caused by these variations were quite small, however, as compared to the deviation caused by GA. Similar differences in GA sensitivity may also exist, and the possibility has to be considered that such inter-experimental variations may create a false impression of an interaction. It is highly improbable, however, that variations of this type should produce a picture as regular as the one obtained. It should also be kept in mind that interexperimental variations necessarily must be included in the error mean square. Consequently, it is concluded that the interactions described above are in all probability real.

IV. Discussion

Possibly, some of the effects caused by GA in the slit pea test may be explained by simple geometrical considerations. If it is assumed that GA acts simply by increasing the length of the inwardly curved arms of the slit sections, it follows that the increase in curvature caused by a certain concentration of GA is roughly inversely proportional to the radius of the curvature caused by IAA simultaneously present. In the slit pea test, the radius of curvature decreases with increasing concentrations of IAA. Accordingly, the GA response must increase with increasing IAA concentrations, which seems to be the case in the present work.

A simple experiment with straight growth of pea epicotyl sections may throw some light upon this question. Sections of 6.1 mm. length were cut

from the apical ends of the third internodes of pea plants cultivated exactly as in the slit pea test. The sections were washed in distilled water and then placed in a 10 p.p.m. solution of GA. After 20 hours the mean length of GA-treated sections was 8.71 mm., that of control sections 8.25 mm., *i.e.* the GA response was 0.46 mm.

The connection between increase in length of the curved part of the section and increase in angle of curvature may be calculated from the formula (cf. Sæbø 1960)

$$(1) \quad \Delta a = \frac{360 \cdot \Delta l}{2 \pi \cdot r}$$

Here, a = angle of curvature, l = length of the curved part considered, and r = radius of curvature.

Strictly speaking, this formula is valid only for circular arcs. But if only the tips of the inwardly curved arms are considered, *i.e.* the portions of the sections being most sensitive to GA (Purves and Hillman 1958), the formula may probably be safely applied.

If the radius of curvature is equal to 5 mm., it is found that an increase in length of the curved part of 0.46 mm. will cause an increase in angle of curvature of some 5° . It is thus possible that an interaction of this purely geometrical type may at least accentuate the effect of GA in the slit pea test.

However, this geometrical interaction cannot explain why pure GA solutions are effective in the slit pea test. Nor can it explain the depressing action of high IAA concentrations on GA responses.

Now it seems to be well established that the presence of auxin is necessary to gibberellin action (Brian and Hemming 1957 and 1958, Kuse 1958, Brian 1959). It also seems to be established that high concentrations of IAA may depress the action of gibberellin on some tissues (Hayashi and Murakami 1953 and 1958, Recalde, Verdejo, and Blesa 1960). These two facts taken together with the purely geometrical type of interaction described above probably explain most of the action of GA in the slit pea test. If the GA response at a fixed level of GA is considered as a function of the concentration of IAA simultaneously present, it is evident that there will be no GA response at zero concentration of IAA. As the IAA concentration increases, the synergism between GA and IAA manifests itself. Finally, at still higher IAA concentrations, IAA will depress the action of GA. In other words, the relation between GA response and IAA concentration at a fixed level of GA will be represented by an optimum curve, the GA effect possibly being accentuated by the geometrical interaction described above. The action of pure GA solutions must then be ascribed to the presence of residual endogenous auxin in the slit sections.

The question of the exact nature of the interaction between GA and IAA in the slit pea test must remain open. For a discussion of the various possible modes of action of GA on pea tissue, see Phillips, Vlitos, and Cutler (1959).

V. Summary

1. GA is active in the slit pea stem curvature test, its principal action being to increase curvatures caused by simultaneously applied IAA.

2. The mode of action of GA in this test is discussed. The action of GA may be explained partly by a purely geometrical interaction, partly by the known interactions between gibberellin and auxin in straight growth of tissue sections.

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Influence of Cupferron on the Geotropic Reaction and the Respiration of Wheat Roots

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Introduction

The course of geotropic bendings of horizontally-placed wheat roots submerged in a water solution has been found to be highly influenced by the supply of oxygen to the root medium (9). In oxygen-deficient solutions the roots are not curved positively as much as in well-aerated solutions. The reason for this was shown to be that the interaction of the positive and negative geotropic bending tendencies determining the course of the reaction was changed in favour of the positive one. The effects caused by oxygen were found to be induced in solutions with high pH values in the root medium and by a low temperature (9, 10). Recently Keitt (4), has confirmed the effects of the latter two factors. In all cases the effect was induced by changes in the root medium and except for the low temperature it could hardly be proved that similar changes take place in the root. In the experiments discussed in this paper an attempt to increase the oxygen supply within the root was made. Kihlman (5, 6) found that some respiratory inhibitors produce an increased frequency of chromosomal aberrations of *Vicia faba* roots. He suggested that this effect is attributable to an increase in the oxygen content of the root, caused by a decrease in oxygen consumption. Thus it seemed interesting to examine the effects of respiratory inhibitors on the geotropic behaviour of wheat roots. Kihlman used cyanide and cupferron, the ammonium salt of nitrosophenylhydroxylamine. However, the geotropic reactions are best distinguished in slightly acid solutions and under these conditions cyanide is very instable. Further, the solutions in which the geotropic reac-

tions take place have to be aerated, which would cause a rapid disappearance of the cyanid from the solution. Therefore cupferron was used in the present experiments. The authors are well aware that this substance may affect the elongation of the roots, *e.g.* have auxin or antiauxin-like effects, and therefore the respiration of the roots was measured in parallel experiments. In this way we hoped to be able to distinguish between effects mediated through the oxygen supply and direct effects on the cell elongation.

Methods

A detailed description of the methods used for the determinations of the geotropic reactions has been published earlier (8, 11). The experimental material, Weibull's Eroica wheat, was soaked in distilled water for 24 hours and germinated on wet filter paper in petri dishes at 25°C. When the roots were 2—3 cm. long the seedlings were placed in small glass tubes. The tubes were placed in perspex holders in such a way that the roots dipped into a nutrient solution. In this position they were left to grow for one night at 15°C. Straight roots were then selected and used for the geotropic experiments. The roots were allowed to curve in a nutrient solution of the following composition KNO_3 10^{-3} , $\text{Ca}(\text{NO}_3)_2$ 10^{-3} M, MgSO_4 $5 \cdot 10^{-4}$ M and a phosphate buffer 10^{-3} M.

The initial pH-value was about 6.0 and an increase to 6.1 or 6.2 could be recorded after the experiments. Cupferron was given dissolved in the nutrient solution. The curvatures were photographically recorded and the angles could be measured directly on the negatives by means of a horizontal microscope of a type similar to that used by Larsen (7). On our instrument the pointer was movable and the scale fixed to the instrument. The measurements of the angles were performed according to the method of Rufelt (8). The angles to the horizontal line were plotted against time, giving time curves. For measurements of the longitudinal growth the negative pictures were projected onto a white paper to give a magnification of 5.4 times and outline drawings were made. On these drawings the position of the commencement of the bending was also determined. Determinations of the effect of cupferron on the respiration of root tips were performed according to the method of Warburg. 10 mm long root tips were used, 40 in every flask. The roots used were grown on moist filter paper for 48 hours.

Experiments

1. Time curves

Cupferron was tested in the concentrations 3×10^{-5} M, 5×10^{-5} M and 10^{-4} M. In preliminary experiments we found that 10^{-5} M had no effect. The time of treatment with cupferron before the start of the geotropic stimulation was different in different experiments, normally 30 minutes or 12 hours. Every experiment was performed with three or four cuvettes; two

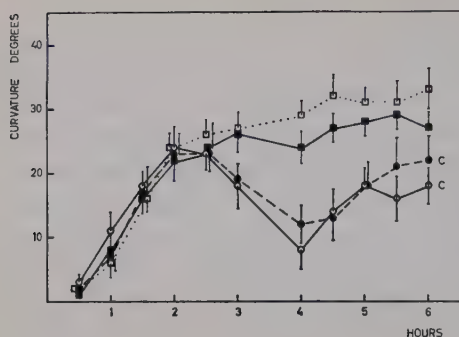


Figure 1. Time curves of the geotropic reaction of wheat roots, treated with cupferron 5×10^{-5} M. The roots were exposed to the inhibitor for 30 minutes before the start of the stimulation. C=Controls.

containing cupferron, and one or two controls containing nutrient solution. Air was always bubbled through the solutions during the experiments. The roots were photographed at 30 minutes intervals for 5 to 6 hours and were then in most cases left for 20 hours. After that time the last picture was taken. From this an idea of the final shape of the curvature could be obtained. After such a time the curvature angle could be measured with only a low degree of accuracy because curvatures in other planes than that perpendicular to the optic axis of the camera had often developed.

Typical time curves of cupferron treated roots are shown in Figure 1. The time curve in its first part is determined by the positive bending tendency only. Normally this initial positive curvature is not affected by cupferron. In some experiments a slight inhibition was found but it was not significant. After 2 to 3 hours straightening begins in the control roots caused by the activity of the negative bending tendency but this is absent or weak in the roots treated with the inhibitor. This is the typical response to the cupferron treatment. In Figure 2 the effect of the time of pretreatment is illustrated. It

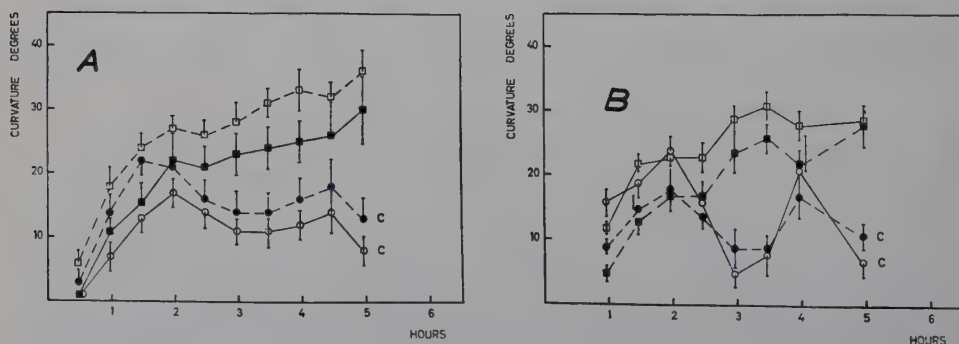


Figure 2. Influence of the time of pretreatment on the geotropic reaction of wheat roots treated with cupferron 3×10^{-5} M. A 12 hours. B 0 hours. C=Controls.

Table 1. *Geotropic curvatures recorded about 24 hours after the commencement of the geotropic stimulation. Curvatures mean deviation of the tip from its original direction. The figures are means for 9 roots.*

Cupferron <i>M</i>	Time of pre- treatment hours	Curvature degrees	
		Treated roots	Control roots
3×10^{-5}	0.5	45 ± 4.9	6 ± 13.3
		38 ± 4.7	—
	12	43 ± 5.2	-13 ± 7.2
		42 ± 4.0	-19 ± 12.7
5×10^{-5}	0	50 ± 4.4	19 ± 14.6
		45 ± 4.8	—
	0.5	42 ± 3.0	-7 ± 7.7
		42 ± 2.1	-42 ± 17.7
	12	5 ± 5.0^1	6 ± 4.1^1
		7 ± 3.4^1	—
1×10^{-4}	0	11 ± 8.1	15 ± 12.0
		-24 ± 11.1	—
	0.5	-3 ± 5.7	-11 ± 10.8
		-26 ± 5.7	—

¹ Measurements after 10 hours (see text).

is obvious that the cupferron effect is essentially the same if the roots are stimulated without pretreatment or if they are exposed to the substance for 12 hours before stimulation. Consequently, it may be deduced that the inhibitor is rapidly absorbed by the roots.

In Table 1 the curvatures recorded after about 24 hours are compiled from a series of experiments. In 3×10^{-5} *M* (all experiments) and 5×10^{-5} *M* (the experiments with a short time of pretreatment), the cupferron treatment makes the curvature more uniform and also more positive. In roots treated for 12 hours in 5×10^{-5} *M* solution the curvature recorded already after 10 hours is low and the variation is rather wide. During the first 6 hours the course of the reaction was not significantly divergent from the controls but a decrease in the positive reaction could possibly be traced. After 24 hours the roots looked "poisoned" and measurements were considered meaningless. The same was true for 10^{-4} *M* solutions both for the reaction of the first 6 hours and the curvature after 24 hours.

2. Longitudinal growth

The length of the curving roots was determined 0, 2, 4 and 6 hours after the start of the stimulation. Table 2 gives the results from the experiment

Table 2. *Influence of cupferron on growth of geotropically curving roots.* The figures are means for 9 roots. Measurements from the same experiment as in Figure 1.

Cupferron <i>M</i>	Growth in mm.		
	0 — 2 hours	0 — 4 hours	0 — 6 hours
5×10^{-5}	0.64 ± 0.08	0.97 ± 0.13	1.28 ± 0.13
	0.53 ± 0.05	0.80 ± 0.07	1.09 ± 0.06
0	0.70 ± 0.05	1.11 ± 0.07	1.63 ± 0.12

Table 3. *Influence of the concentration of cupferron and the time of pretreatment on growth of geotropically curving roots.* The figures are means for 9 roots.

Cupferron <i>M</i>	Time of pre-treatment hours	Growth/6 hours	
		Treated roots	Control roots
3×10^{-5}	0.5	1.90 ± 0.10	1.93 ± 0.13
		1.40 ± 0.07	
5×10^{-5}	0.5	1.28 ± 0.13	1.63 ± 0.12
		1.09 ± 0.06	
1×10^{-4}	0.5	1.01 ± 0.07	1.78 ± 0.11
		0.74 ± 0.05	
5×10^{-5}	12	0.53 ± 0.11	1.85 ± 0.13
		0.41 ± 0.07	

illustrated in figure 1. Table 3 illustrates the effects of the cupferron concentration and the time of pretreatment. A decrease in growth of the roots treated with the inhibitor was always found. It was larger with increased concentration and was most pronounced in experiments with 12 hours pretreatment.

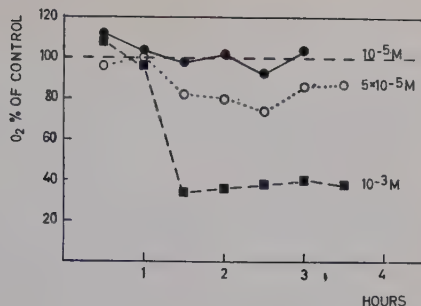
3. Localization of the start of the bending

The distance from the root apex to the place of the start of the geotropic bending was determined in 38 cuvettes. The mean value was found to be 1.0—1.5 mm. in 32 cuvettes; and 1.6—2.0 in 6. Differences between treated and control roots were not found. The conclusion is that the bending starts in a distance of about 1.5 mm. from the tip and the place is not changed by the addition of cupferron.

4. The respiration of root tips of wheat

Typical results are shown by figure 3. The concentrations 10^{-5} , 3×10^{-5} , 5×10^{-5} , 10^{-4} , and 10^{-3} *M* were tested. In all concentrations except 10^{-5} *M* an inhibition of the oxygen consumption was found. This was fully developed

Figure 3. Influence of cupferron on oxygen consumption of wheat roots.



after 30 minutes, which indicates that cupferron is rapidly absorbed by the root. The inhibition increased with the concentration of the inhibitor. Thus the influence on the geotropic reaction takes place in the same concentrations as the influence on the respiration.

Discussion

The experiments show that the course of the geotropic reaction is changed in the same way by cupferron as it is changed by a high supply of oxygen *e.g.* the roots bend more positively. On the other hand, growth was decreased by cupferron while it was increased by oxygen (9). However this does not imply that the effect of cupferron essentially differs from that of oxygen. The inhibition of the negative geotropic reaction seems to be complete already in the lowest active concentration, while growth was gradually inhibited by an increasing concentration of cupferron. If growth is measured as increase in length of the roots the result is mainly determined by the cell elongation. This process is most rapid in the basal part of the elongation zone (3). The results of Burström were confirmed by Hejnowicz (2) who found a very low and constant rate of elongation in the zone 0.2 to 0.8 mm. from the apex. The velocity was gradually increased and reached its highest value in the basal part of the elongation zone. This was found to be 4 to 5 mm. in length. According to our measurements the geotropic bending starts about 1.5 mm. from the apex. Consequently the curvature studied is not formed by the cells giving the most rapid elongation and changes in growth caused in the curving part can be completely masked by the growth changes in more basal parts of the roots. This might explain, firstly, why the increasing inhibition of growth with increasing concentration of cupferron is not connected to a change of the positive geotropic curvature; and, secondly, why the effect on the negative reaction is the same in all solutions, even if the effect on

growth increases with increasing concentration of the inhibitor. This also implies that growth and geotropic reaction cannot be compared directly and that the identical effect on geotropic reaction elicited by oxygen and cupferron need not necessarily be accompanied by identical effects on elongation growth.

Rufelt (11, 12) has suggested that both the positive and the negative geotropic reaction are induced by growth inhibitors. The positive reaction is due to an accumulation of IAA on the lower side of the root: the negative bendings are elicited by some growth inhibitor, which is not IAA and is probably a substance which is more labile than IAA. The effect of oxygen on the negative reaction was assumed to be caused by an oxidation of this hypothetical substance, accumulated in the upper side. Accordingly, the effect of cupferron can be explained as due to the increase in the oxygen supply, caused by the fact that influence on respiration and on the negative geotropic reaction is found to start at the same concentration level. The other possibility that cupferron counteracts the hypothetical substance in a way similar to that of phenylisobutyric acid cannot be excluded, but then it must be considered as a strange coincidence that effects on geotropism and on respiration start at the same level. Audus and Brownbridge (1) have found that in pea roots the positive geotropic reaction is followed by a growth inhibition on the upper side. They suggest that a growth inhibitor accumulated on the lower side spreads to the upper one and that consequently the inhibition on the lower and the upper sides are caused by the same inhibitor. That cupferron affects the negative reaction extensively cannot be explained by the hypothesis of Audus and Brownbridge.

Of course, cupferron has more radical effects besides the inhibition of the negative geotropic reaction. These effects are evident in all experiments with the most concentrated solution 10^{-4} M, and in 5×10^{-5} M with 12 hours pretreatment. The effects can be assumed to be caused by the inhibition of respiration and can only be described as "poisonous". Presumably they can be considered to develop more slowly than the effect on the geotropic reaction, an opinion which is supported by the fact that they are more pronounced after a long cupferron treatment.

Thus our interpretation of the effect of cupferron on wheat roots is that the inhibitor produces an inhibition of respiration, which is followed by a general decrease in various physiological processes. This "general poisoning" is manifested as a decrease in growth and in the positive as well as the negative geotropic reaction. Before this "poisonous" effect is fully developed an increase in the oxygen content induces a selective inhibition of the negative geotropic reaction. The increase in oxygen content is caused by a decrease in the consumption of oxygen in respiration.

Summary

The effects of the respiratory inhibitor, cupferron, upon respiration, growth and the geotropic reactions of wheat roots were studied. In the minimum concentrations inhibiting respiration, growth is decreased. The positive geotropic reaction is not affected while the negative one is inhibited. In higher concentrations all the processes are inhibited. The peculiar effect on the negative reaction is assumed to take place via an increase in the oxygen concentration caused by the inhibition of respiration.

The possibility of drawing false conclusions in comparing growth and geotropic reaction is discussed.

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On Carbon Dioxide Accumulation in Roots and Its Effect on Respiration Measurements

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Many investigations on plant respiration include simultaneous determinations of carbon dioxide output and oxygen uptake, allowing the calculation of the exchange ratio between the two gases: $\frac{\Delta \text{CO}_2}{-\Delta \text{O}_2}$. This quotient, referred to in the following as the Gas Quotient (G.Q.), often supplies important information for the evaluation of the processes responsible for the gas exchange.

According to the literature, gas quotient values show a wide range of variation. The variations can be related to type and quantity of the respiratory material as well as to environmental conditions (Ruhland and Ramshorn 1938, Ramshorn 1957, 1958, 1959, Ziegler 1957). Extremely high G.Q. values are generally found when the external oxygen concentration is low. However, in certain cases, when the oxygen concentration is not a limiting factor, similar, high G.Q. values are nevertheless found. This occurrence of extremely high G.Q. values in oxygen-rich environments has lately been confirmed by the author during investigations on respiration in intact corn and tomato roots (Jensen 1960). The present series of experiments was designed to further elucidate these findings.

Material and Methods

Tomato plants of the variety "Moneymaker" were grown in constantly aerated nutrient solutions until 4 to 6 weeks old. Intact roots were used for the experiments as in previous studies (Jensen 1957, 1960). The carbon dioxide release and oxygen

consumption was determined by the tonometer method (Jensen 1957), which had been modified slightly. The pH measurements of the circulating nutrient solution in the tonometer apparatus have been especially improved by inserting a remote-control glass- and calomel-electrode unit in the system. In this manner changes in the pH values of the nutrient solution passing the roots could be recorded with an accuracy of ± 0.005 pH unit throughout the experiment. This procedure gave an improved accuracy in the determination of the amount of carbon dioxide released from the roots since it allows a calculation of the total amount of carbon dioxide (dissolved + bound in the nutrient solution at 10 to 12 minute intervals, simultaneously with the tonometer determinations of dissolved (free) carbon dioxide and oxygen. In earlier investigations with the tonometer method it was possible to determine only the total amount of carbon dioxide in the circulating solution at the start and at the conclusion of an experiment generally consisting of six single determinations of free carbon dioxide and six determinations of oxygen (made within a period of 65–70 minutes).

All experiments were carried out at the same temperature, $20 \pm 0.1^\circ\text{C}$. The light intensity at the top leaves of the experimental plants was approximately 35,000 lux.

Results

The results are presented in graphs, the contents of oxygen and carbon dioxide in the circulating nutrient solution being plotted as a function of time. Significant changes in the contents of these gases provide an expression of root respiration. The respiratory rate can be estimated directly from the slope of the time curves and is further expressed by the gradient, here defined as the gas increment or diminution per minute multiplied by 100. In the following we refer to the consumption coefficient for oxygen and the release coefficient for carbon dioxide as b_{ox} and b_{cd} respectively.

In planning the investigations three experimental schemes were considered and used for a total of 26 experiments or series of experiments. Among these three, each representing one of the said schemes, will be described in detail.

Experiment 1, Figure 1. This experiment was carried out in exactly the same way as that previously used by the author. In preparation for the experiment the root was placed in the tonometer apparatus for 20 minutes surrounded by undisturbed, noncirculating nutrient solution. This solution was discarded immediately before the start of the experiment and replaced by a fresh, oxygen-rich (in equilibrium with atmospheric air) solution which, at time zero, was brought into circulation in the apparatus. The results show that the oxygen uptake over the entire experimental period proceeds at a constant rate while the carbon dioxide release during the first 25 minute interval (t_1) proceeds at a considerably higher rate than in the following interval (t_2). Calculation of b_{ox} and b_{cd} values for each of the two intervals,

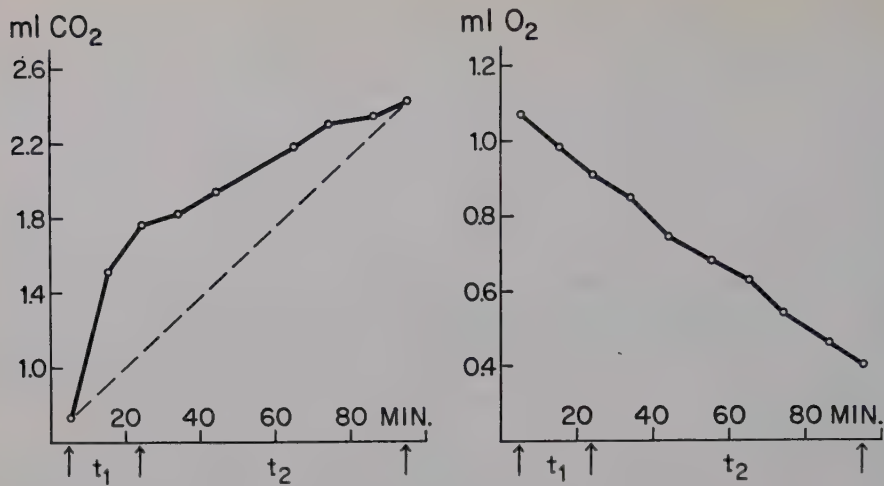


Figure 1. *Respiration of intact tomato roots.* Ordinates: ml total CO₂ (free+bound), or ml O₂, in the circulating nutrient solution of the tonometer apparatus. *t*₁ and *t*₂ initial and final part of experimental time. The broken line illustrates the curve for changes in total carbon dioxide (free+bound) as estimated by earlier technique.

*t*₁ and *t*₂, results in the gradients listed for experiment 1 in Table 1. This table also contains gas quotients calculated from the gradients.

In the previously mentioned 1960-experiments it was possible to determine the amount of carbon dioxide released from the roots only by means of the values for the total content of carbon dioxide calculated for the solution at the start and at the conclusion of an experiment. In order to justify the use of this procedure it is necessary to assume that the roots release carbon

Table 1. *Respiration determinations with intact tomato roots.* *b*_{ox} and *b*_{cd} calculated rates for oxygen consumption and carbon dioxide output, respectively. *G.Q.*, gas quotient. *t*₁ and *t*₂, initial and final part of experimental period and *t* total experimental period.

Experiment No.	Pretreatment	Interval	b _{cd}	b _{ox}	G. Q.
1	Undisturbed solution	t ₁	5.60	0.67	8.4
		t ₂	0.99	0.67	1.5
2 A	do	t ₁	10.20	(1.36)	7.5
		t ₂	1.10	1.36	0.8
2 B	do	t ₁	7.56	(1.50)	5.0
		t ₂	2.31	1.50	1.5
3 A	do	t ₁	3.64	0.99	3.7
		t ₂	1.56	0.99	1.6
3 B	Circulating solution	t	0.90	1.05	0.9
3 C		do	t	0.98	1.00

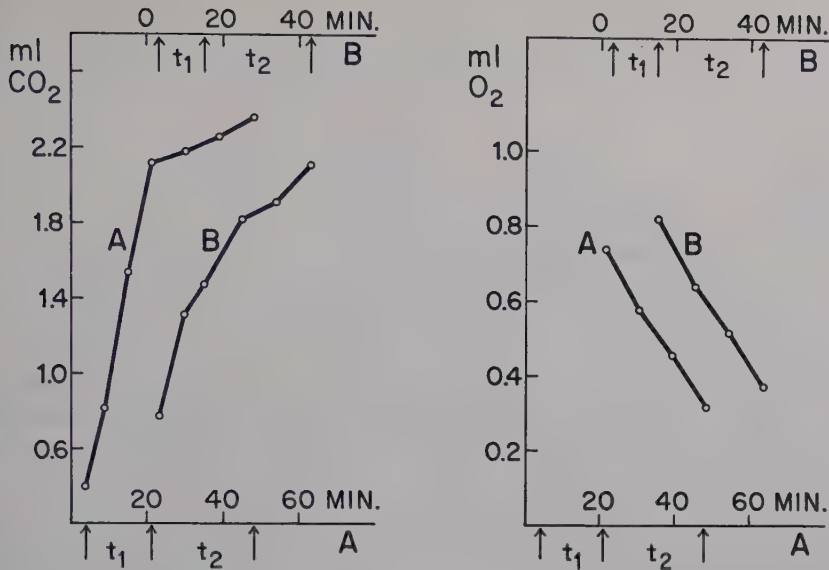


Figure 2. *Respiration of intact tomato roots kept in undisturbed nutrient solution until start of measurements. Two experiments performed with one plant, the abscissas and curves being denoted with corresponding letters.*

dioxide at a constant rate as indicated by the broken line in Figure 1. From the carbon dioxide curve obtained by the modified technique it is obvious that this assumption does not prove to be correct under the conditions of the experiment. It was decided to investigate (in experiments of type 2 and 3) whether the changing rate of carbon dioxide release found in experiment 1 could be correlated in some way with the fact that the root remained in undisturbed solution prior to the respiration determinations.

Experiment 2, Figure 2. Two experiments (2 A and 2 B) were carried out with the same plant. Preceding the first experiment, and in between the experiments, the root was placed in undisturbed nutrient solution. This pre-treatment had a duration of 20 minutes in experiment 2 A. Immediately after the conclusion of this experiment the circulation was stopped and the root remained in the undisturbed solution for 50 minutes. After this period the nutrient solution was exchanged during a 10 minute period in such a manner that very little movement occurred in the immediate vicinity of the root before circulation commenced for experiment 2 B.

Results of the two experiments (2 A and 2 B) are presented in Figure 2 and Table 1. They show, as did the results of experiment 1, that during the early part of the experiments the root releases carbon dioxide at a higher

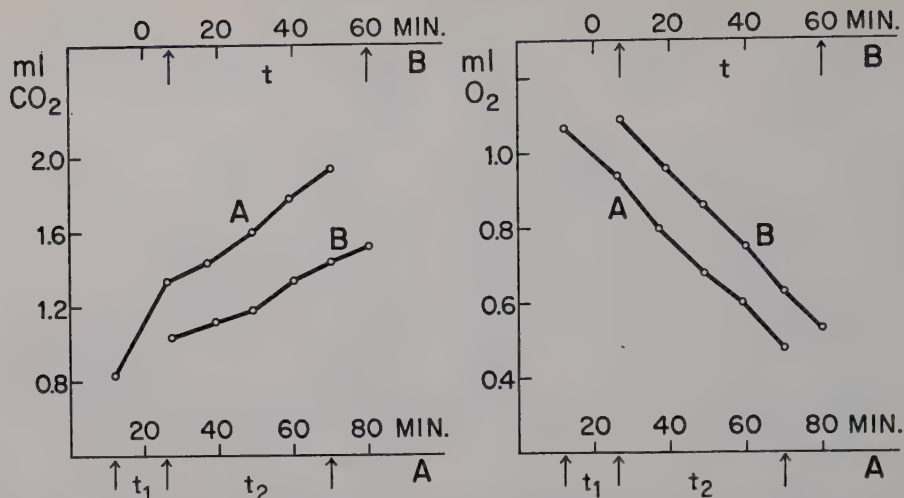


Figure 3. *Respiration of intact tomato roots.* Results from two experiments, A and B, with the same plant. Prior to experiment A the root was kept in undisturbed nutrient solution for 20 minutes, prior to B the solution was circulated for 20 minutes before the start of the measurements.

rate than later, and that the rate of oxygen consumption is constant throughout the experimental period. They also show that it is possible to reproduce the uneven rates of carbon dioxide release in experiments with one and the same plant if the root is kept in undisturbed nutrient solution in between the experiments.

Experiment 3, Figure 3. To further investigate the problem of the changing rate of carbon dioxide release, three experiments were carried out with another plant (3 A, 3 B, 3 C). The schedule for the root treatment was: undisturbed solution 20 minutes, then exchange of solution and start of circulation which was continued throughout experiment 3 A and throughout the 20 minute interval between this experiment and the next (3 B): immediately before the start of 3 B the nutrient solution was again replaced. In between experiment 3 B and 3 C the solution around the root was also kept moving and replaced by a fresh solution just before the start of experiment 3 C.

Results of the 3 experiments (3 A, B, C) are given in Table 1. Experiments 3 B and 3 C gave identical results and only those from 3 B have been plotted in the graph (Figure 3). The root in experiment 3 A had been surrounded with undisturbed solution until the start of the experiment and released its carbon dioxide at changing rates as did the roots in experiments 1 and 2. In

experiments 3 B and 3 C, however, the root has been situated in circulating nutrient solution prior to the measurements and the carbon dioxide release during the experiment proceeded at a constant rate.

Discussion

A survey of the figures in Table 1 indicates that the rate of oxygen uptake is constant, or approximately constant, for different periods of the same experiment, and for the same plant in different experiments. The figures for carbon dioxide output, however, vary over a wide range. The rate varies with time during individual experiments, and also is altered from experiment to experiment with the same plant. This variation is correlated with the nature of the root treatment prior to the respiration determinations. When the root is kept in undisturbed solution the variation is high, but a pretreatment in circulating solution diminishes the variations, within individual experiments as well as within series of experiments with the same plant. Under these experimental conditions the carbon dioxide output proceeds with a constancy similar to that of the oxygen consumption.

The extraordinarily high rate of carbon dioxide release found at first when roots are transferred from undisturbed to circulating medium is a new feature in root respiration revealed by the modified tonometer technique. The reason for its occurrence may be discussed on the basis of the following assumptions. 1) All of the carbon dioxide causing the high rate of output is produced during the period during which the measurements are performed, or 2) Part of the carbon dioxide is produced prior to this period, to be accumulated and subsequently released when circulation and measurements are started.

The former hypothesis does not seem probable. The only way to explain a surplus production of carbon dioxide causing high G.Q.-values observed in the first part of the experimental period is to assume that anaerobic respiration is going on simultaneously with the aerobic one during this period. However, the oxygen concentration in the solution around the root cells is at its maximum at the beginning of the experiment, just after the exchange of the nutrient solution. It must be considered unlikely that anaerobic conditions in the root tissues should exist during the first half of the experiment and not during the second one when even a part of the oxygen is consumed.

It may be noted here that an increase in the gas exchange of the roots may be expected when circulation starts in the tonometer apparatus. The movement of the nutrient solution, which is slightly pulsating due to the operation of the pump, causes the root branches to move to and fro bending slightly from

moment to moment. Audus (1935, 1939, 1940, 1941, 1946) and Godwin (1935) have observed that gentle bending of leaves can introduce a considerable increase in respiration, and they registered this by means of the carbon dioxide output. Similar effects have been demonstrated in experiments with leaf blades and other kinds of plant tissues by Barker 1935 (Potato tubers), Stiles and Dent 1947 (Beetroots), and Kahl 1951 (Lettuce leaves). In the tonometer experiments it was not possible to decide whether or not an Audus effect occurred in the roots. However, it is unlikely that this effect, if present, should affect only the carbon dioxide output, and then only during the first part of the experiments.

The second hypothesis, i.e., that an excess of carbon dioxide is produced before the measurements are made, to be accumulated and then released during the first measurements, seems to fit in with current knowledge acquired during the present study and those of other authors. Past experience has shown that a period in which the solution in the tonometer apparatus is left undisturbed, is required for the rapid release of carbon dioxide in the beginning of the measurement series to take place. It is quite possible that carbon dioxide accumulates in the root tissue during the stay in the undisturbed solution. An accumulation of this gas in compact plant organs such as potato tubers and apples has been demonstrated to take place by Magness (1920), Denny (1946 a, b, 1947) and was also observed in seeds of various species (Geiger 1928). In the present discussion it is important to note that Burström (1959) found an accumulation of carbon dioxide to take place in wheat roots when intact as well as when detached. The meristematic tissues were particularly rich in accumulated gas.

Changes in the experimental conditions following the start of the circulating pump will result in a more or less abrupt release of the accumulated carbon dioxide. Firstly, the circulation of the solution will alter the gradients improving the diffusion possibilities for the gas; secondly, the aforesaid moving and bending of root branches, compressing some cells and stretching others, will create a pulsating mass movement of gas molecules. The release of the "extra" carbon dioxide starts upon the onset of circulation and this is confirmed by the findings. It seems well established that the high G.Q.-values found immediately determinations begin are caused by a sudden release of carbon dioxide left over from an earlier production.

Summarizing the discussion of the experimental results we may draw the general conclusion that measurements of oxygen uptake provide the most satisfactory basis for determinations of the rate of root respiration, at least in the case of short time experiments. This main result of the investigations strongly support the ideas of Laties, who in 1957 came to a similar conclusion based on a review of the literature. The preference of oxygen measurements

must be of particular importance in the case of ecological experiments with roots growing in soil, since it is highly probable that conditions favouring high gas quotients are present in the root environments and in the root tissues.

Summary

Root respiration in intact tomato roots was studied by simultaneous determinations of carbon dioxide release and oxygen consumption. When the root remained in undisturbed nutrient solution for a short time preceeding the determinations (20 minutes), the rate of carbon dioxide release was higher during the initial experimental period (20—30 minutes) than during the subsequent period. The oxygen uptake proceeded at a constant rate throughout the experiment. The hypothesis is advanced that the "extra" carbon dioxide release during the initial experimental period is due to a delayed release of carbon dioxide produced and accumulated in the root tissue prior to the experiment. It is concluded that determination of oxygen consumption is the most reliable method of measuring root respiration in short time experiments.

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Components in the Uptake and Transport of High Accumulative Ions in Wheat

By

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Introduction

Most investigators working with ion uptake in plants are now of the opinion that this uptake consists of different components, which are usually divided into active and passive ones. Accumulation and bleeding are considered to be active components supposed to require energy from metabolic processes. Ion uptake components based on purely physical phenomena as diffusion, adsorption and mass flow are classed among the passive ones. Concerning the size and the importance of those different components, however, opinions diverge strongly.

During the last years much interest has been taken in the importance of transpiration for ion uptake (Hylmö 1953, 1955, 1958, Brouwer 1953, 1954, 1956, van den Honert *et al.* 1955, Kylin and Hylmö 1957, Russell and Shorrocks 1958, 1959, Pettersson 1960). In nearly all these investigations it has been demonstrated that ion uptake increases with transpiration. Although the experimental results are in good agreement with each other, the ways of interpreting the relation between ion and water uptake are very divergent. Hylmö (1953) assumed that ions were passively transported from the medium to the shoot with the mass flow mediated by the transpiration stream. Brouwer (1953, 1954, 1956) explained his similar experimental results with a theory that the conductivity for water and ions in the root might increase on the enhanced suction in the root caused by increased transpiration. Russell and Shorrocks (1959) were of the opinion that transpiration influenced the ion uptake only when the concentration in the stele was so high that it

worked against accumulation. In that case increased transpiration would promote the removal of ions and thus stimulate the bleeding. Recently Pettersson (1960) has presented experimental results indicating that the relation between transpiration and ion uptake is more complicated. According to Pettersson transpiration may be of importance to the ion transport in the mass flow and act as a bleeding-stimulating factor as well.

The present investigation is intended to be a supplement to earlier works dealing almost exclusively with ions which are only slightly accumulated in the plant tissues. In order to investigate whether the rules and relations which have been demonstrated for the absorption of low accumulative ions are valid also for those ions which are accumulated to a higher degree, a series of experiments has been performed where the uptake of potassium and nitrate has been determined at varying transpiration rates and medium concentrations.

Materials and Methods

Wheat of Weibull's "Eroica" variety was used for the investigations. The seeds were germinated by soaking in tap water overnight and then spread out on moist filter paper in Petri dishes, which were then placed in a dark room at 22°C for two days. After this period the seedlings were transferred to a nutrient solution. The seedlings were mounted on round perspex disks, seventeen seedlings on each disk. Three such plant groups were then placed in a 1-litre beaker containing 800 ml nutrient solution.

Nutrient solutions with varying nitrate and potassium concentrations were used in the different experimental series. The basis of these variations was a standard nutrient solution with the following composition (Kylin and Hylmö 1957): 1 mM $\text{Ca}(\text{NO}_3)_2$, 1 mM KH_2PO_4 , 1 mM KCl, 0.5 mM MgSO_4 , 0.5 mM Na_2HPO_4 , 0.01 mM ferric citrate, 0.1 μM MnSO_4 , 0.1 μM H_3BO_3 . When it was desirable to obtain plant material with a lower nitrate content, $\text{Ca}(\text{NO}_3)_2$ in the standard solution was partially substituted by CaCl_2 . A nutrient solution where KCl was reduced or excluded and where KH_2PO_4 partially was replaced by NaH_2PO_4 was used to obtain plants with a low potassium content. In connection with the experiments account will be given of the potassium and nitrate concentrations of the nutrient solutions in the different cases.

The beakers were put in a photothermostat where light was supplied continuously by four fluorescent lamps (about 3500 lux). The temperature was kept at 24°C. Continuous aeration of the solution was arranged, and water losses were made up for every day. Every three days the nutrient solution in the beakers was renewed.

The experiments were performed when the plants had grown for 7 days in the nutrient solution. One plant group from each beaker was used for determination of the initial ionic status. Each one of the other plant groups was placed in a test tube containing 150 ml. solution. The solutions used in the experiments were composed as the nutrient solution mentioned above but the amounts of nitrate and potassium were varied. In the nitrate uptake experiments solutions were used where the nitrate

concentrations were 1, 2, 5, 10, 20, 40, and 80 mM. The five last mentioned concentrations were attained through addition of NaNO_3 . In the corresponding potassium experiments the potassium concentrations were 1, 5, 10, 20, and 30 mM. The potassium content was here increased by adding KCl.

The test tubes with the plants were put into two thermostats, one dark, the other supplied with light from two 500 watt incandescent bulbs at an intensity of approximately 9000 lux. In a few experiments another thermostat with light, in which the relative air humidity could be increased to about 90 per cent, was used. The temperature was kept at 24°C.

During the experimental period, 2—24 hours, the solutions in the test tubes were usually aerated. In other cases parallel experimental series with and without aeration were performed.

Transpiration and water uptake were measured by weighing according to Hylmö (1953, Table 1). Through experiments with test tubes where the plants were replaced by pieces cut out of a rubber tube, the loss due to the evaporation from the free water surface in the test tubes was determined.

At the end of the experimental period the plants were divided into roots and shoots and dried for three hours at 105°C, after which the dry weight was determined.

The nitrate analyses were made according to a colorimetric method after separation on ion exchangers, which has been described in detail by Stoy (1955). Some experimental series were also analyzed for the total nitrogen content according to the Kjeldahl method. For the potassium analyses the plant material was incinerated four hours at 470°C, after which the ashes were dissolved in hydrochloric acid. Then the potassium concentration of the solutions was determined by means of the Eppendorf flame photometer. Some potassium analyses were performed directly on the nutrient solutions used in the experiments.

Experiments

One of the difficulties in the present work was to obtain plant material where the initial status of the ion which was to be examined was so even that the amount of ions which the plants managed to take up during a few hours could easily be discerned. If the wheat plants were cultivated in the standard nutrient solution described above, the initial potassium and nitrate levels varied within too wide limits. When the plants were to be used in experiments with nitrate uptake, the nitrate concentration in the nutrient solution was reduced to 0.1 mM, which gave a low and comparatively even initial nitrate status in roots and shoots.

It proved to be more difficult to obtain plant material with an even potassium level. If the potassium concentration in the nutrient solution was reduced to 0.3 mM, the potassium content in the roots became satisfactorily even, but the content in the shoots still varied so much that small increases could not be discerned. In those cases where the potassium uptake from more diluted (<10 mM) solutions was to be examined and where the amount of ions absorbed during the experimental period was accordingly small, the author had to use the method of analyzing the nutrient solutions at the start and at the end of the experiments instead of analyzing the plant material. This method has of course the disadvantage that it does not give any information about the proportions of the absorbed ions remaining in the roots and of those moving into the shoots.

Beside the uptake of ions in the root there exists a leakage, a stream of ions in the opposite direction out into the medium. Some preliminary experiments, with high and low potassium status plants, cultivated on 1 mM respectively 0.3 mM potassium gave information about the occurrence and the extent of this leakage. Aerated and unaerated experimental solutions with the potassium concentration varying from 1 to 10 mM were used. In the high salt plants the uptake exceeded the leakage only when the plants were put into aerated solutions with higher concentrations (> 2 mM). In the more diluted and the unaerated solutions the leakage exceeded the uptake in all experiments. However, the leakage dominated less at high than at low transpiration. As to the low salt plants the uptake exceeded the leakage even when unaerated solutions with low potassium concentrations were used. The fact that the leakage has proportionally small importance in low salt plants justifies the use of such plants in the experimental series described below.

In order to determine the extent of the nitrate assimilation some of the series among the nitrate uptake experiments were analyzed for total nitrogen content. It turned out that about one third of the amount of nitrate taken up and retained in the roots was assimilated, since the increase in the amount of assimilated nitrogen was about half the increase in nitrate content. In the shoots the assimilation was greater and amounted even to half the total increase. No differences could be discovered between the extent of the nitrate assimilation in light and in darkness. Nor was the degree of aeration in the experimental solutions of any importance to the assimilation. Moreover, the nitrate assimilation was independent of the transpiration intensity.

a. *The relation between ion uptake and water transport*

The results of an experiment where the potassium uptake was determined at three different transpiration intensities are shown in Figure 1. In this experiment the plants were placed in three different thermostats: one dark

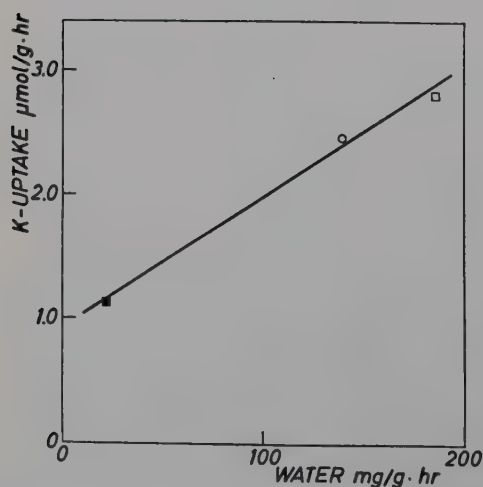


Figure 1. *Net uptake of potassium by wheat plants at varying rates of water transport. Squares — relative air humidity 50 per cent. Circle — relative air humidity 90 per cent. Filled symbol — darkness. Open symbols — 9000 lux. — Potassium concentration of the medium 20 mM. All values refer to fresh weight of whole plant.*

and with a relative humidity of about 50 per cent, two supplied with light and with relative humidities of 90 and 50 per cent respectively. The plants used in the experiment were cultivated on a nutrient solution with the potassium concentration 0.3 mM, and the experimental solution was 20 mM in respect to potassium. The experimental period was six hours. It appears from Figure 1 that the potassium uptake increased with increasing water transport. The three dots lie along a straight line. As the increased air humidity only decreased the transpiration by 25 per cent, the two upper dots unfortunately lie rather close to each other. However, through varying the transpiration in different ways and with different plant material other investigators have obtained straight regression lines for the relation between ion and water uptake with many dots spread along a larger part of the regression line (Brouwer 1956, Pettersson 1960, van den Honert *et al.* 1955, recalculated by Pettersson 1960, figure 3).

The straight regression lines for the relation between ion and water uptake, which are obtained in spite of the fact that transpiration is varied with different methods, indicate that the variations in light and air humidity in short-time experiments do not influence the ion uptake in any other way than through the changed transpiration intensity. Hylm  (1953) obtained an equal change in the calcium and chloride uptake regardless of whether he varied the transpiration through changed air humidity or light strength. Russell and Shorrocks (1959) showed through experiments with plants which were prepared with light of different intensities that the increase in the uptake of rubidium and phosphate at increased light strength was a direct result of the increased transpiration rate and had nothing to do with any changed metabolic conditions.

The proofs for a linear connection between ion and water uptake mentioned above appear quite satisfying, and in all the following experiments I have used only two different transpiration levels, one low caused by darkness and one high obtained by an illumination of the shoots with 9000 lux. Other factors, *e.g.*, air humidity, temperature, and aeration, have been constant and similar in the two cases.

Table 1 is based on experiments performed with plants cultivated on a nutrient solution with the nitrate concentration 0.1 mM. In these experiments the aim was to find out how the ion absorption was influenced by the concentration of the medium. The medium concentrations examined in the experiments were 1, 40, and 80 mM. The experiments were performed partly with and partly without aeration in the test tubes during the experimental period, which was six hours in all cases. Analyses for total nitrogen showed that the increase in the amount of assimilated nitrogen in the roots at the three medium concentrations used were 0.20, 0.35, and 0.50 $\mu\text{mol/g}$ plant

Table 1. Increase in nitrate content in wheat plants at varying water transport and medium concentration.

Medium	NO ₃ - conc. medium mM	Low transpiration			High transpiration			$\frac{k}{C_M}$ (Influxcoeff.)	k mM
		Water transport root mg/g plant. hr	Increase in the nitrate content in the root $\mu\text{mol/g}$ plant. hr	Increase in the nitrate content in the shoot $\mu\text{mol/g}$ plant. hr	Water transport root mg/g plant. hr	Increase in the nitrate content in the root $\mu\text{mol/g}$ plant. hr	Increase in the nitrate content in the shoot $\mu\text{mol/g}$ plant. hr		
un-aerated	1	15	0.29	0.57	86	0.34	0.80	3.2	3.8
		19	0.35	0.50	115	0.41	0.72	2.3	
		15	0.27	0.54	87	0.36	0.91	5.1	
		19	0.57	0.67	98	0.63	1.08	5.2	
		19	0.31	0.51	95	0.58	0.77	3.4	
	40	21	0.35	0.35	83	0.49	0.57	3.5	6.8
		14	0.51	0.39	104	0.63	0.97	0.16	
		16	0.33	0.52	84	0.47	1.16	0.24	
		18	0.50	0.73	86	0.66	1.23	0.18	
		12	0.70	0.71	66	0.84	1.06	0.16	
	80	16	0.83	0.69	85	0.96	1.18	0.15	16
		25	0.69	0.80	65	0.71	1.04	0.15	
		20	0.65	0.59	79	0.81	0.92	0.14	
		16	0.88	0.74	88	0.96	2.30	0.27	
		14	0.64	0.57	60	0.47	1.41	0.23	
aerated	1	15	0.68	0.77	89	0.76	1.74	0.16	2.0
		15	1.00	0.78	61	1.18	1.50	0.20	
		15	1.00	0.75	75	1.13	1.51	0.16	
		20	0.59	0.80	117	0.57	0.98	1.9	
		21	0.64	0.71	115	0.57	0.90	2.0	
	40	12	1.22	0.53	83	0.90	1.15	0.22	8.4
		12	1.38	1.03	72	1.26	1.72	0.29	
		12	1.55	0.71	90	1.61	1.06	0.11	
		14	2.00	0.98	59	2.02	1.58	0.17	
		17	2.54	0.82	55	2.38	1.65	0.27	
	80	12	1.22	0.53	83	0.90	1.15	0.22	17.6
		12	1.38	1.03	72	1.26	1.72	0.29	
		12	1.55	0.71	90	1.61	1.06	0.11	
		14	2.00	0.98	59	2.02	1.58	0.17	
		17	2.54	0.82	55	2.38	1.65	0.27	

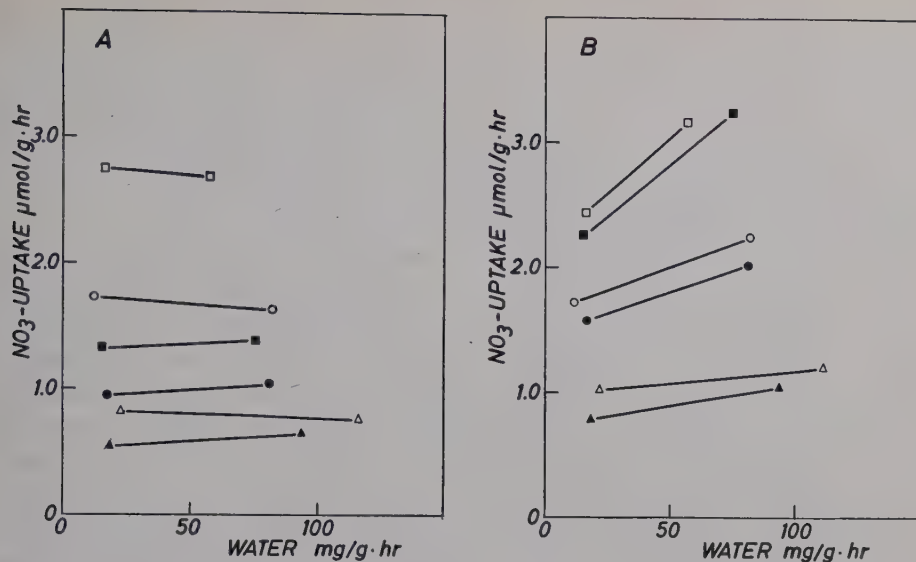


Figure 2. Net uptake of nitrate by wheat plants at varying rates of water transport and medium concentrations. Values corrected for nitrate assimilation.

A. Nitrate retention in the roots.

B. Nitrate transport to the shoots.

Triangles — nitrate concentration of the medium 1 mM

Circles — " " " " " 40 mM

Squares — " " " " " 80 mM

Open symbols — medium aerated. Filled symbols — medium unaerated.

All values refer to fresh weight of whole plant.

and hour respectively. If these values are added to the figures in Table 1 concerning increase in nitrate content in the root, one gets the part of the total amount of the absorbed ions which is retained in the root. The means of the values for nitrate retention in Table 1 with correction for the nitrate assimilation mentioned above have been plotted in Figure 2 A. Apparently the ion retention in the root increases with increased medium concentration, but it seems to be uninfluenced by the water transport. The small increases with the transpiration in unaerated roots and the decreases in aerated, which can be seen in Figure 2 A, have not been found again in later experiments with other medium concentrations. The importance of the aeration to the absorption of nitrate ions in the roots is very great. At the medium concentration 1 mM the ion retention was 30 per cent greater with than without aeration. With 40 and 80 mM solution the corresponding increase with aeration was 60 and 100 per cent.

The transport of nitrate to the shoot is, contrary to the ion retention in the root, highly influenced by the transpiration, which appears from Figure 2 B. This figure is based on the values in Table 1 with correction for the nitrate assimilation of the shoot. The increase in the amount of assimilated nitrogen at the three medium concentrations employed was 0.25, 0.95, and 1.55 $\mu\text{mol/g}$ plant and hour. As mentioned above, the extent of nitrate assimilation was independent of the transpiration intensity.

The ion transport to the shoot can be divided into two components. One component, obtained by extrapolating the ion-water uptake curve to the ordinate, is independent of the water flow through the plant. The other component, by Pettersson (1960) called "excess uptake", increases rectilinearly with the water transport to the shoot. The transpiration-independent component in the nitrate transport to the shoots is like the nitrate retention in the roots dependent on the degree of aeration in the medium. The transport to the shoots, however, is not influenced to the same degree as the ion retention in the roots. While the aeration of a 40 mM nitrate solution increased the nitrate retention in the roots about 60 per cent, the corresponding increase in the transport to the shoots at imaginary zero water transport is only about 15 per cent. Nor did an increased medium concentration cause such a great increase in the transpiration-independent component in the nitrate transport to the shoots as it did in the ion retention in the roots.

The ion transport to the shoot follows a line $y=kx+l$, where l , the ordinate at zero water uptake, represents the component of ions transported to the shoot independent of water flow. The slope of the line, k , *i.e.*, the part of the ion transport dependent on transpiration (kx) divided by the corresponding amount of water (x), represents the concentration in the water flow-induced component in the sap stream. From Figure 2 B it is evident that this slope increases with increasing medium concentration. Hylmö (1953) introduced for the relation between k and the medium concentration (C_M) the term influx coefficient. This term has turned out to be somewhat inappropriate and in many cases it has been misunderstood by other investigators.

In Table 1 a column with the values of $\frac{k}{C_M}$ (the influx coefficients) is inserted. It is apparent that the values of $\frac{k}{C_M}$ from the experiments with 1 mM test solutions are considerably higher than those based on experiments with 40 and 80 mM test solutions, which, on the other hand, agree well with each other. This is also the case with aerated and unaerated solutions.

In order to examine the influence that transpiration exerts upon potassium uptake at different medium concentrations a series of experiments was performed corresponding to the nitrate uptake experiments described above.

Table 2. *Absorption of potassium in wheat plants at varying water transport and medium concentration.*

Medium	K- conc. of the medium mM	Low transpiration		High transpiration		$\frac{k}{C_M}$ (Influxcoeff.)	k mM
		Water transport through the root mg/g plant. hr	Potassium absorbtion $\mu\text{mol/g}$ plant. hr	Water transport through the root mg/g plant. hr	Potassium absorbtion $\mu\text{mol/g}$ plant. hr		
unaerated	1	27	0.41	109	0.51	1.2	1.9
		26	0.21	150	0.51	2.5	
	5	24	0.61	116	1.02	1.0	0.9
		27	0.39	166	0.93	0.8	
	20	25	0.78	85	1.26	0.40	8.0
		24	0.56	102	0.90	4.4	
aerated	1	27	0.72	147	1.35	5.2	3.9
		28	0.70	142	1.15	4.0	
		26	0.86	161	1.15	2.2	
		23	0.71	116	1.38	1.4	
	5	27	0.91	149	1.61	1.1	1.2
		23	0.96	171	1.84	1.2	
		32	1.29	156	2.30	0.41	
	20	24	1.48	186	2.41	0.29	0.43
		19	0.98	205	2.40	0.38	
		25	1.27	166	3.19	0.68	

The plant material was cultivated on a nutrient solution which was 0.3 mM in respect to potassium. In the test solutions the potassium concentrations were 1, 5, and 20 mM. The results of the experiments are shown in Table 2, and the means of these values are plotted in Figure 3. As the potassium absorption from the 1 and 5 mM solutions has been determined through medium analyses, which do not admit any division of the uptake into root and shoot components, for the sake of unity the total potassium uptake has been inserted into Table 2 concerning the experiments with 20 mM solution too. In the last mentioned case analyses on the plant material showed that the transpiration influences the potassium uptake in the same way as the nitrate uptake. The retention of potassium in the root remains unchanged at increased transpiration, as the corresponding component in the nitrate uptake. Accordingly the slope of the regression line for the relation between the total potassium uptake of the plant and the water flow represents the concentration of the transpiration-induced component in the sap stream to the shoot. The values of the relation between the angle coefficient k and the medium

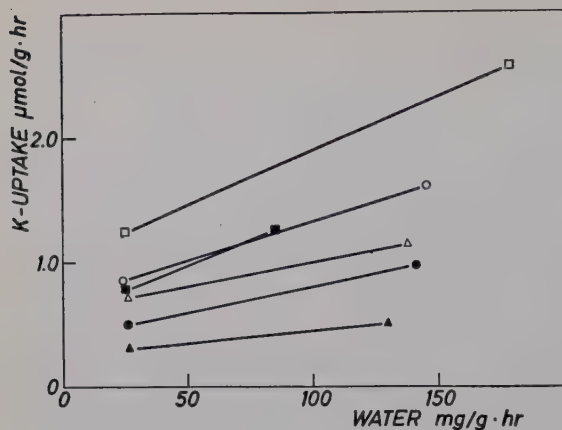


Figure 3. Net uptake of potassium by wheat plants at varying rates of water transport and medium concentrations.

Triangles — potassium concentration of the medium 1 mM.

Circles — potassium concentration of the medium 5 mM.

Squares — potassium concentration of the medium 20 mM.

Open symbols — medium aerated.

Filled symbols — medium unaerated.

All values refer to fresh weight of whole plant.

concentration are inserted in Table 2. As was the case with nitrate these values are highest at low medium concentrations. From Figure 3 it appears also that the part of the potassium uptake which is independent of transpiration is highly influenced by the degree of aeration in the medium.

The concentrations of the transpiration induced component in the sap stream to the shoot ($=k$) are inserted in the columns to the right in the Tables 1 and 2. Hylmö (1953) called this concentration "the true concentration of the transpiration stream". It is obvious that the concentration in this component increases with increasing medium concentration. In order to

Figure 4. The relation between the concentration of the water flow-induced component in the sap stream (k) and the medium concentration (C_M).

Open circles — potassium. Filled circles — nitrate.

The broken line represents a value of $\frac{k}{C_M}$ (influx coefficient) of about 0.17.

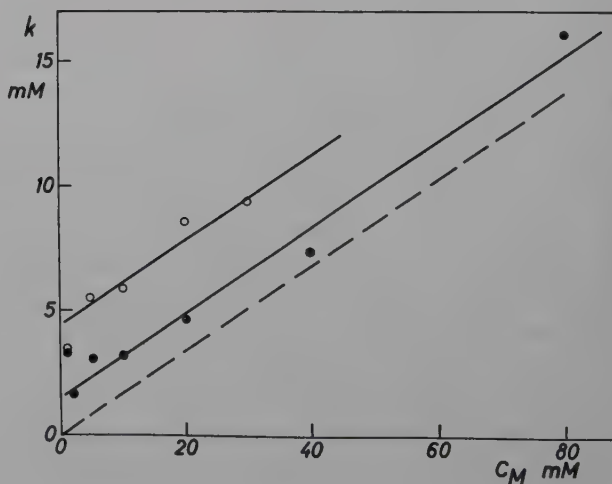


Table 3. *Influx coefficients calculated from values of ion uptake as noted in the literature.*

Ion	Medium conc. mM	Influx coeff.	Plant material	Investigator
K	0.8	1.1	beans	Wright 1939
	1	3.9	wheat	Kihlman-Falk 1961
	5	1.2	wheat	Kihlman-Falk 1961
	6.8	1.1	beans	Freeland 1937
	6.8	0.16	maize	Freeland 1937
	7.2	1.4	Sanchezia	Schmidt 1936
	20	0.43	wheat	Kihlman-Falk 1961
	78	0.05	maize	Böttcher & Behling 1939
Rb	1	1.2	barley	Russell & Shorrocks 1959
Ca	1	0.56	peas	Hylmö 1953
	1.5	1.1	beans	Wright 1939
	5	0.56	peas	Hylmö 1953
	6.8	0.17	beans	Freeland 1937
	10.5	1.25	Sanchezia	Schmidt 1936
	120	0.02	maize	Böttcher & Behling 1939
NO ₃	1	3.8	wheat	Kihlman-Falk 1961
	4	0.12	beans	Wright 1939
	21	0.42	Sanchezia	Schmidt 1936
	40	0.17	wheat	Kihlman-Falk 1961
	80	0.20	wheat	Kihlman-Falk 1961
PO ₄	1	0.9	barley	Russell & Shorrocks 1959
	6.8	0.50	maize	Freeland 1937
	6.8	0.21	beans	Freeland 1937
	93	0.02	maize	Böttcher & Behling 1939
SO ₄	0.25	0.54	sunflower	Pettersson 1960
	0.5	0.27	sunflower	Pettersson 1960
	0.5	0.26	wheat	Kylin & Hylmö 1957
	5	0.25	wheat	Kylin & Hylmö 1957
	5	0.21	sunflower	Pettersson 1960
	50	0.11	sunflower	Pettersson 1960
Cl	2	0.61	peas	Hylmö 1953
	8	0.58	peas	Hylmö 1953
	10	0.14	broad beans	Brouwer 1954
	32	0.49	peas	Hylmö 1953
Griseofulvin	3 mg/l	0.15	beans	Crowdy <i>et al.</i> 1956
	6	0.29	beans	Crowdy <i>et al.</i> 1956

elucidate the relation between these two concentrations a series of experiments was performed with medium concentrations other than those mentioned above. The results are shown in Figure 4. The values for the nitrate and potassium uptake fall along two straight lines running parallel to each other. The potassium line lies above the nitrate line. Moreover the lines are parallel to an imaginary line through the origin, representing a value of $\frac{k}{C_M}$ (the influx coefficient) of about 0.17.

b. The time course of ion absorption

In a series of experiments the time courses of the nitrate and potassium absorption were investigated. The plant material used was "low salt plants" cultivated as described above on media containing 0.1 mM nitrate and 0.3 mM potassium. Test solutions which were 40 mM in respect to nitrate were used in the nitrate uptake experiments. Plant groups were analyzed 4, 6, 12, and 24 hours after the start of the experiments.

The total nitrate absorption increased during the experimental period rectilinearly with the time (Figure 5 A). However, if the total ion uptake is divided into ion retention in the root and transport to the shoot, the nature of the absorption becomes clearer. The time course of the nitrate retention in the root indicates an ion uptake composed of two phases: an initial rapid phase, already completed two hours after the start of the experiment (cf. Figure 6) and a subsequent slower phase in the absorption. During this later period the ion content in the roots increased at a constant rate. The transport of nitrate to the shoot (Figure 5 A) proceeded at a constant rate during the experimental period.

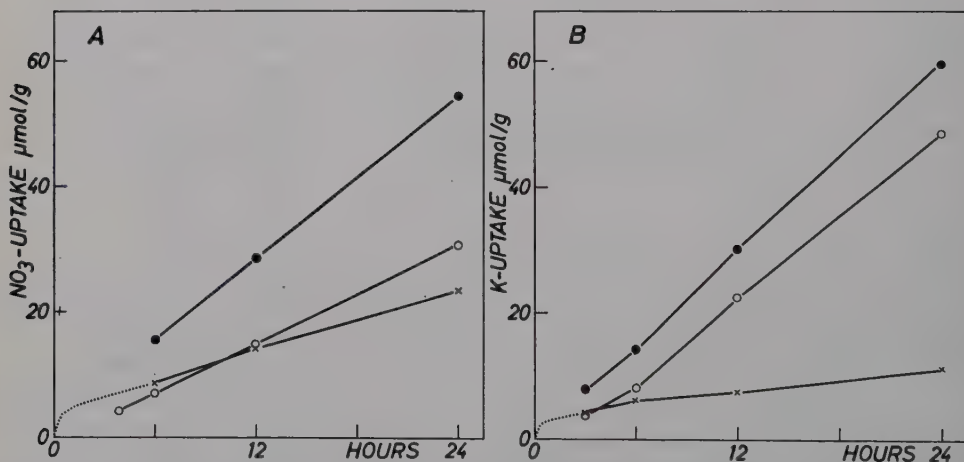


Figure 5. Time course of nitrate and potassium absorption at high transpiration rate.

A. Nitrate absorption from a medium with the nitrate concentration 40 mM. The values corrected for nitrate assimilation.

B. Potassium absorption from a medium with the potassium concentration 20 mM.

Filled circles — total absorption.

Crosses — ion retention in the roots.

Open circles — ion transport to the shoots.

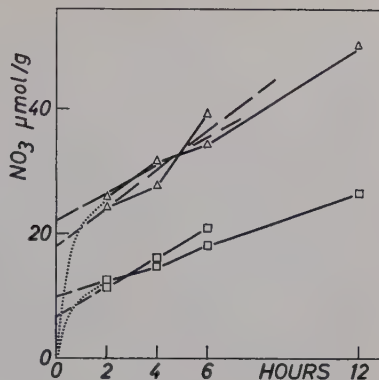
All values refer to fresh weight of whole plant.

Figure 6. Time course of the increase in nitrate content in the roots.

Squares — nitrate concentration of the medium 40 mM.

Triangles — nitrate concentration of the medium 80 mM.

The values refer to fresh weight of root.



Corresponding experiments with potassium gave similar results. The test solution was here 20 mM in respect to potassium. The experimental times were about the same as in the nitrate experiments described above. The total potassium absorption as the nitrate uptake increased rectilinearly with the time (Figure 5 B). However, there seems to exist a slight difference between them. Whereas an extrapolation downwards of the "potassium line" seems to go nearly through the origin, the extrapolated "nitrate line" cuts off a part of the ordinate. If the total uptake of potassium is divided into root- and shoot components as has been done in Figure 5 B, one obtains a potassium retention with a time course quite like the time course of the nitrate retention in Figure 5 A. The transport of potassium to the shoot has a time course similar to the course of the corresponding component in Figure 5 A too, but the rate of the ion transport does not reach a constant value until after some hours. Evidently the difference between the total uptake of nitrate and potassium mentioned above is due to this slower transport of potassium to the shoot at the beginning of the experiments.

The rapid initial phase found in the nitrate and potassium absorption of the roots is in agreement with the results obtained by many investigators working with different ions. This phenomenon has been interpreted as a passive diffusion of ions between the medium and the apparent free space (AFS) of the root. (Hope and Stevens 1952, Butler 1953, Hylmö 1953, Briggs and Robertsson 1957). Depending on ion species adsorption sites may be of more or less importance within the AFS. Positive ions are adsorbed to a greater extent than negative ones, which will be clear from a comparison between values of AFS calculated below from experiments with nitrate and potassium uptake. By extrapolating the ion uptake of the roots back to the

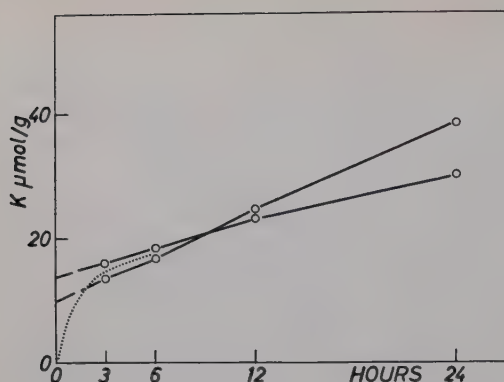


Figure 7. Time course of the increase in potassium content in the roots.

Potassium concentration of the medium 20 mM.

The values refer to fresh weight of root.

ordinate, one obtains the amount of ions absorbed in the AFS. The size of AFS in per cent of the root volume can then be calculated from the equation

$$\frac{\mu\text{mol initial uptake/g fresh weight}}{\text{ambient concentration (mM)}} \times 100 = \text{AFS \%}$$

As the nitrate ions are assimilated and thereby removed from the free space, it is not possible to calculate the size of AFS from Figure 5 A, which illustrates the total increase in the nitrate retention including the assimilated part. Calculations of AFS would give too high values here. In Figure 6, however, only the time course of the increase of the nitrate content in the roots has been plotted and thus it is possible to calculate the AFS from these values. Figure 6 is based on four experiments, two with 40 and two with 80 mM test solution. In the former case AFS is calculated to 18 and 25 per cent in the latter to 23 and 27 per cent.

The time course of the increase of the potassium content in roots is shown in Figure 7. If the AFS is calculated here from the initial rapid uptake, one obtains 50 and 70 per cent. These high values can be explained by the fact that positive ions are adsorbed to a greater extent than negative ones which gives a wrong picture of the AFS.

Discussion

In 1958 Hylmö briefly mentioned that one had to expect the bleeding to be a dominating component in the transport of high accumulative ions as potassium and nitrate to the shoot. This has proved to be correct. It is usually accepted that the ion uptake, which is going on in the plant when the transpiration is zero is composed of diffusion into the free space and adsorption

to electrically charged points there and of accumulation and bleeding implying active uptake driven by metabolic processes. Hylmö (1953) determined the size of this transpiration-independent part of the ion uptake through extrapolation of the regression line for ion and water uptake and through experiments with excised roots. With a 4–5 mM CaCl_2 experimental solution this uptake of calcium was about $0.07 \mu\text{mol/g}$ fresh weight and hour. The corresponding chloride uptake was $0.05 \mu\text{mol/g}$ fresh weight and hour. Of the ions thus absorbed 50 to 75 per cent were retained in the root. The rest obviously represents the bleeding to the shoot. As expected the corresponding values were much higher in the present experiments with potassium and nitrate uptake. At an imagined zero transpiration $1.8 \mu\text{mol}$ nitrate/g fresh weight and hour were absorbed from an aerated 1 mM nitrate solution. Of this amount $0.8 \mu\text{mol}$ was retained in the root and $1.0 \mu\text{mol}$ was transported to the shoot. The corresponding uptake of potassium from a 5 mM potassium solution was about $0.7 \mu\text{mol/g}$ fresh weight and hour. If it is supposed that the proportion of the ions which is retained in the root and of those which is transported to the shoot is the same for potassium as for the other ions mentioned above, this implies that the bleeding component is about ten times as great in the uptake of potassium compared with the corresponding component in the calcium uptake. As to the nitrate uptake the bleeding component seems to be still greater.

In addition to the great transpiration-independent potassium and nitrate absorption there exists an uptake which is directly dependent on transpiration. Experimental solutions with rather high concentrations of the ion in question must of course be used in order to be able to detect this component in the ion uptake. The theory of a relation between water and ion uptake was criticized by van den Honert *et al.* (1955), who, however, used very diluted solutions in their own experiments. Thus the transpiration-independent component in the ion absorption was very dominating in their experiments and consequently they could not discern the relatively small water flow-dependent component in the ion uptake.

As was briefly mentioned in the introduction, different authors have very divergent opinions in their interpretation of the existing relation between water and ion uptake. Hylmö (1953) based his opinion about a purely passive component in the ion uptake caused by the mass flow, which is mediated by the transpiration stream, on experiments with calcium and chloride in pea plants. "The true concentration" of the transpiration stream, in which ions absorbed through diffusion, adsorption and accumulation were not included, turned out to be independent of the rate of water transport through the plant but dependent on the medium concentration in relation to which it was always diluted. Hylmö found that the "influx coefficient", *i.e.*, the

relation between "the true concentration" of the transpiration stream and the medium concentration, was the same at all tested medium concentrations (1—16 mM CaCl_2). Further, the same values were obtained for the different ions. These facts Hylmö considered as a proof that the transpiration-dependent component in the ion uptake was a solely passive process, as he found it difficult to understand how the constant value of the influx coefficient could be coupled with an active process. Hylmö explained the fact that "the true concentration" of the transpiration stream was lower than the medium concentration by the occurrence of a sieve mechanism in the root which maintains a uniform dilution of the transpiration stream at all medium concentrations.

Previously Brouwer (1953, 1954) held that the component in the ion absorption which increased with increasing water transport was solely active. In a later paper (1956), however, Brouwer admitted that in addition to the large water flow-dependent component conditioned by metabolic activity there probably exists a smaller passive component directly dependent on the rate of the water transport through the plant. On the other hand, according to Hylmö (1958) a smaller part of the water-linked ion transport is due to bleeding, which is probably stimulated when ions which originally were actively accumulated by the xylem are passively carried along with the transpiration stream. Thus the difference between the ways in which the two authors interpret the importance of active and passive components in the ion uptake is now quantitative rather than qualitative.

Russell and Shorrocks (1959) and later Russell and Barber (1960) have criticized the hypotheses for ion uptake which were presented by Hylmö (1953) and Kylin and Hylmö (1957). However, their criticism is based to a great extent on a misunderstanding. Russell and Barber wonder that Hylmö can say that the rectilinear relation between water and ion transport implies that "the true concentration" of the transpiration stream is the same for all rates of water transport, when "on the graphic representations of his results, the lines showing the relationship between absorption and transpiration seldom passed through the origin and suggest that appreciable absorption would have occurred when transpiration was zero. Thus, the concentration in the transpiration stream did not remain constant but decreased with increased transpiration." Obviously Russell and Barber have not understood that with the term "the true concentration" Hylmö does not mean the total ion concentration in the sap stream to the shoot but only the concentration of the transpiration-dependent component in this stream. Hylmö has never denied that in addition to the passive ion uptake there exists an active one, which especially at low transpiration rate can cause the concentration of the sap stream to exceed the medium concentration considerably.

As Russell and Shorrocks cannot accept the occurrence of a considerable passive transport of ions with the mass flow against the high concentrations which can exist in the stele, they maintain that the increase in ion uptake at increased transpiration rate must depend on stimulated bleeding. Moreover, they are of the opinion that such a stimulation only exists when the concentration in the stele becomes so high that it works against the active accumulation. Similar theories on the importance of the transpiration for ion uptake have earlier been presented by Broyer and Hoagland (1943).

Hylmö (1953) based his opinion about a transpiration-dependent passive component in the uptake among other things on the discovery that the influx coefficients for calcium and chloride uptake were constant within the concentration range tested. Later investigations performed at this institute have shown that the relation between ion uptake and transpiration is somewhat more complicated than Hylmö had earlier reason to suppose. Pettersson (1960) investigated sulphate absorption at different transpiration rates in young sunflower plants. He found that the influx coefficients at low medium concentrations were considerably higher than when more concentrated experimental solutions were used. Sulphate solutions of 0.25, 0.50, 5, and 50 mM concentration were tested and the influx coefficients obtained were in the different cases 0.54, 0.27, 0.21, and 0.11 respectively. Pettersson interpreted his experimental results with a hypothesis that the transpiration-linked component of the ion transport was not of a purely passive nature regulated solely by a sieve mechanism in the root but that this component also included a transpiration-stimulated bleeding. At higher medium concentrations this bleeding would be negligible in comparison with pure mass flow, implying that the influx coefficients here would have about the same value. In diluted media, however, the addition of the transpiration-stimulated bleeding, which is proportionally great in these cases, to the mass flow might give high influx coefficients.

The results of the present author from investigations of potassium and nitrate absorption in wheat are in good agreement with Pettersson's experimental results. The values of $\frac{k}{C_M}$, the influx coefficients, in Table 1 show an obvious increasing tendency at lower medium concentrations, while at higher concentrations they have a quite constant value about 0.20. The potassium experiments in Table 2 do not extend over as large a concentration range as the nitrate experiments, but here too, one can discern a similar tendency in the relation between influx coefficients and medium concentrations. However, the influx coefficients for potassium are somewhat higher throughout than the corresponding nitrate coefficients.

An analysis of Figure 4 provides an explanation of the varying influx

coefficients. In Figure 4 one axis represents the concentration of the transpiration-induced component in the sap stream (k), in other words the concentration of "the excess uptake", and the other axis the concentration of the medium (C_M). If "the excess uptake" were purely passive, the relation between those two concentrations ought to give a straight line through the origin, implying that the relation $\frac{k}{C_M}$, the influx coefficient, was constant and independent of the medium concentration. A hypothetical line of that kind representing an influx coefficient of about 0.17 has been drawn in Figure 4 (broken line). The nitrate as well as the potassium line are parallel to this hypothetical line. If "the excess uptake" is composed of a passive component regulated by some sieve mechanism in the root and an active component, stimulated bleeding, the former component might be represented by the region below the broken line. Thus, the concentration in this passive component would be about 20 per cent of the medium concentration independent of the ion in question and at all tested medium concentrations. A stimulated bleeding might then be added to this passive component. The region between the broken line and the nitrate and potassium lines would represent the stimulated bleeding if the hypotheses above are true. In that case this component would be different from the passive one in being uninfluenced by the medium concentration. This independence would imply that an equal amount of ions would be absorbed through this mechanism at all medium concentrations at least within the tested concentration range. On the other hand, the stimulated bleeding would be of varying degree for different ions, which is natural concerning an active mechanism. According to Figure 4 it would be three times as great for potassium as for nitrate. The high influx coefficients obtained at lower concentrations of the medium might be explained by the proportionally greater importance which the stimulated bleeding would have at lower medium concentrations. Further, the differences in influx coefficients existing between different ions might be explained through the proportionally unequal importance which the stimulated bleeding would have beside the passive component in "the excess uptake".

There are many older investigations dealing with discovered relations between ion uptake and transpiration (Schmidt 1936, Freeland 1937, Bötticher and Behling 1939, Phillis and Mason 1940). Usually the authors only point out the existence of an evident relation between ion and water absorption, but they do not try to explain the cause of this relationship. As the information about the experiments, for instance about the medium concentrations, in those older investigations is meagre and sometimes difficult to interpret, it is often difficult to compare those old experimental results with

the newer ones discussed above. In Table 3 some influx coefficients calculated from older and newer experiments are collected. As a very varying plant material has been used in these investigations not only concerning plant species but also with respect to cultivation and age, too far-reaching conclusions should not be drawn from the material in Table 3. However, certain tendencies can be discerned. The coefficients usually seem to decrease with increasing medium concentration. Moreover, the coefficients for the absorption of the high accumulative potassium ion are rather high compared with the other ions. The influx coefficient for potassium calculated on Freeland's (1937) experiments with beans in a 6.8 mM potassium solution is 1.1, while the corresponding coefficients for phosphate and calcium are 0.20 and 0.17. The last mentioned values are in good agreement with Pettersson's influx coefficients for sulphate uptake and the potassium coefficient agrees with the experimental results of the present investigation. On the other hand, Freeland's experiments with maize give a considerably lower influx coefficient for the potassium uptake (0.16). Bötticher and Behling (1939) investigated the absorption of potassium, calcium and phosphate in maize from highly concentrated media. Those experiments give very low influx coefficients too (0.04 for potassium and 0.02 for calcium and phosphate). Thus it seems as if the transpiration was of less importance to the ion uptake in maize than in other tested plant species. Brouwer (1953), too, obtained divergent results with maize, as the ion uptake at low transpiration rates here seemed to be quite uninfluenced by the water flow. The ion uptake experiments which Brouwer performed with broad beans give normal influx coefficients (0.14 at the medium concentration 10 mM). Furthermore, it is of especial interest in Table 3, that the influx coefficients of the neutral substance griseofulvin in experiments performed by Crowdy *et al.* (1956), are of the same magnitude as the other coefficients.

The existence of a space in the root accessible to free ion diffusion is now considered to be nearly proved. What is still discussed, however, is the size and localization of this free space. Whereas some investigators (Levitt 1957, Brouwer 1959) maintain that the AFS is localized in the cell walls, others (Hope and Stevens 1952, Butler 1953, Kramer 1957, Briggs *et al.* 1957) include the cytoplasm too.

The author's investigations of the time course of the nitrate absorption in wheat roots give AFS values varying from 18 to 27 per cent. Those values are in good agreement with many existing calculations of the AFS. Butler (1953) determined with chloride uptake experiments the AFS in wheat roots to about 25 per cent, and Kylin and Hylmö (1957), working with sulphate absorption in the same material, got AFS values of about 27 per cent. In barley roots Epstein discovered a "true outer space" of 23 per cent. Further-

more, Pettersson (1960) obtained AFS values of about 20 per cent in young sunflower roots. Those high values of AFS have been criticized by Levitt (1957), who considers the method of blotting the roots between filter paper or something similar, which is used in all the works cited above, to be unsatisfactory. According to Levitt a medium film adhering to the root surface would not be removed through the blotting procedure, thereby causing too high AFS values. Levitt has corrected Butler's AFS values for a surface film of 0.02 mm and thus reduced the free space to 8—10 per cent of the root volume. According to Levitt this value would correspond to the volume of the cell walls in the root and thus support the theory that the free space only includes the cell walls and not the cytoplasm.

In a recent paper Ingelsten and Hylmö (1961) present new data from determinations of the free space volume in roots. In their experiments errors due to a surface film have been eliminated by centrifugation. They conclude that the AFS in wheat roots is 11—14 per cent, which is in good agreement with the values calculated by Levitt.

Summary

Through investigations of the potassium and nitrate absorption in intact wheat plants at varying medium concentrations and transpiration rates the following components of the ion uptake have been distinguished:

1. A passive rapidly completed initial phase in the ion uptake of the roots consisting of:
 - a. Diffusion into the free space, AFS, of the root. From nitrate uptake experiments the size of AFS has been calculated to about 24 per cent of the root volume.
 - b. Adsorption to electrically charged points in the free space of the root. This adsorption is negligible for anions as nitrate. For cations as potassium, however, it is important, and it causes the wrong picture of the free space (50—70 per cent) which is obtained from investigations of the initial phase in the potassium uptake.
2. A slower accumulation of potassium and nitrate ions in the root cells. This accumulation proceeds at a constant rate, and it is large compared with the corresponding uptake of, for instance, calcium and chloride ions.
3. Active transport of ions from root to shoot causing the bleeding stream. At increased transpiration rate the transport of ions to the shoot increases, and a part of this increase can be attributed to stimulated bleeding. At low medium concentrations this stimulated bleeding seems to be of proportionally greater importance than at higher concentrations.

4. Passive transport of potassium and nitrate ions to the shoot through mass flow mediated by the transpiration stream. This component is considered to be of great importance at high medium concentrations where it seems to dominate over the bleeding component.

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Action Spectrum for the Elimination of the Lag Phase in Chlorophyll Formation in Previously Dark Grown Leaves of Wheat

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A short light impulse given to a dark grown leaf, followed by a stay in darkness for 4—6 hours induces a high rate of chlorophyll formation during a subsequent period of continuous irradiation (Withrow *et al.* 1956, Wolff *et al.* 1957, Virgin 1958). The difference in the rate of pigment formation as compared to that in a non-preirradiated leaf is mainly restricted to the first 1—3 hours of irradiation, during which time the non-preirradiated leaf shows a lag phase in the formation which disappears after the pretreatment with a short light impulse.

The effect of the light consists of an influence on the formation of the precursor to chlorophyll α , protochlorophyll (Wolff *et al.* 1957, Virgin 1958).

Preliminary reports on the effect of different qualities of light to cause this disappearance of the lag phase indicate that the mechanism for the reaction is with all probability coupled to the general red and far-red light absorbing pigment system, first reported by Borthwick *et al.* (1952b), acting in so many phases of plant development (see Liverman 1960, Mohr 1960).

In the present paper report is given on the action spectrum for this light effect on dark grown leaves of wheat. To obtain this spectrum, dark grown leaves were irradiated with monochromatic energies of known amounts, kept in darkness for five hours and thereafter irradiated for two hours with white light. The amount of chlorophyll a formed during this period of irradiation will be a measure on the effect of the preirradiation. Consideration is mainly

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paid to the effect of red and far-red light — the low energy reaction (Mohr 1960). Whether a high energy reaction where blue and far-red light is also involved has not been closer investigated.

Material and Method

Plant material

The material for the investigation consisted of seven days old leaves of wheat grown at 22°C in complete darkness in nutrient solution according to Virgin (1958). For the irradiation experiments pieces of the leaves, four centimeters long, cut 2.5 centimeters from the tip were used. During irradiation and pigment determination the leaves, 45 at a time, were inserted in a holder which could be irradiated from two opposite sides and provided with devices for aeration, see Figure 1.

Irradiation

The monochromatic light was produced — if not otherwise stated — by a Bausch & Lomb grating monochromator, focal length 500 millimeters, with a tungsten band lamp as light source fed with voltage-stabilized current. The half band width was kept at 10 mμ for wave lengths between 550 mμ to 800 mμ and at 15 mμ for those below 550 mμ. The stray light is small in this type of monochromator, but for safety proper coloured glass filters with limited transmission ranges were used together with the monochromator. Only the first order spectrum was used. The intensity of the irradiation was adjusted by means of the V-shaped slides at the entrance and exit slits.

The light energy was measured by means of a Kipp & Zonen thermopile in connection with a mirror galvanometer, calibrated in absolute units at the university of Utrecht.

Through the inset of proper lenses at the exit slit, the image of the grating could be placed ten centimeters from the slit with the size of two by two centimeters, just large enough to completely cover the opening of the sample holder.

The continuous irradiation which was always started five hours after the end of the short monochromatic light impulse consisted of white light from fluorescent lamps, type Philips TL 65W/29, giving an illuminance of 4000 lux at the place for the sample.

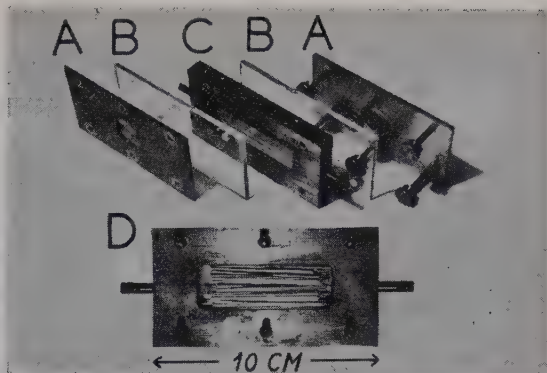
During all treatments, except for the preirradiation, the leaves were continuously aerated by humidified air. The temperature was kept at 22°C if not otherwise stated. All dark treatments of the leaves, *i.e.* the inserting in the holders were performed in weak green safe light according to Withrow and Price (1957).

Pigment determination

The chlorophyll concentration in the leaves was obtained by determining the light absorption of the leaves in the holder *in situ*. Objections can be raised against such a procedure as differences in thickness and scattering phenomena in the leaves will affect the absorption, particularly at low and high pigment concentrations. To

Figure 1. The sample holder used for the irradiation experiments.

- A. Cover plates of brass with holes (diam. 2 cm).
- B. Perspex plates.
- C. Center part of the holder with opening for the leaves and with in- and outlets for aeration. The depth of the opening could be varied by the inset of small plates of Perspex.
- D. The center part from above with leaves.



get around these difficulties a standard curve was plotted for the extinction of the leaves in the holder *vs.* the real chlorophyll contents in leaves treated in the same way as the leaves in the holder. These last-mentioned values for the chlorophyll were obtained by means of extraction and spectrophotometric determination according to Virgin (1956). The curve thus obtained is seen in Figure 2. In this curve the actual chlorophyll concentration could then be found for all values for the extinction of the leaves in the holder. The tedious work of pigment extraction of a very great amount of samples could then become restricted to the establishment of this relationship. The chlorophyll determination at the actual experiments were thus reduced to a simple photoelectric determination. By using 45 leaves in the holder, evenly distributed in about three layers in the holder, there were comparatively small differences in light absorption of samples treated in the same way. In 40 samples (45 leaves each) the chlorophyll *a* formed during two hours of irradiation

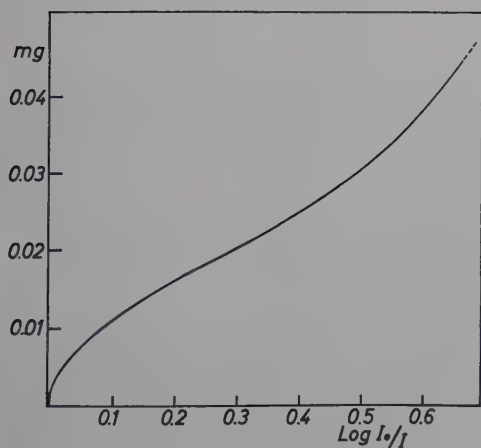


Figure 2. The relationship between the absolute chlorophyll *a* contents in mg/g of fresh weight (ordinate) and the extinction values which will be obtained for the leaves when placed in the irradiation chamber (abscissa).

(without preirradiation) amounted to 0.0120 mg. per gram of fresh weight with a standard deviation (S) of ± 0.0002 , calculated according to the formula

$$S = \pm \sqrt{\frac{\sum x^2}{N(N-1)}}$$

where x =deviations from arithmetic mean, and N =total number of samples. For the determination of the absorption, filtered light within the range 600—700 m μ was used, covering the red absorption peaks of the chlorophylls. The filters used consisted of two "Calflex" heat reflexion interference filters together with a two millimeter thick coloured glass filter (Chance OR 2). The intensity of the transmitted light was measured by means of a photocell-galvanometer system, calibrated for linearity.

At the determination, the following procedure was followed. After the stay in darkness for five hours after the short monochromatic preirradiation, the holder with the leaves were irradiated for five minutes with 500 lux incandescent light. During this time in light practically all the protochlorophyll in the leaf, formed during the dark period, will be transformed into chlorophyll *a* (Smith and Benitez 1954). The transmission of the sample at this stage was put equal to 100. After two hours of continuous irradiation the transmission was measured again. In order to be able to measure many sample holders with the same irradiation and photocell device, the sample could be replaced by a standard filter. In this way, the original light intensity, used for the different samples at the first measurements could be easily reset at the second measurement after two hours of irradiation. The actual chlorophyll concentration could be read in the standard curve, as mentioned above.

Experimental

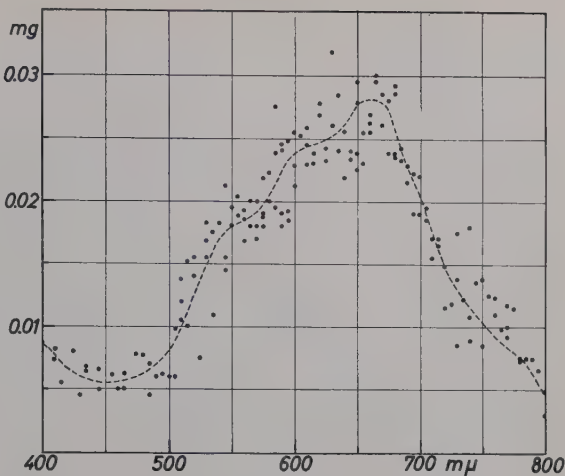
1. *The action spectrum for the promotion of chlorophyll formation*

In a dark grown leaf of wheat, which is exposed to light, the full speed of the process of greening does not start until after one to three hours of continuous irradiation at room temperature (Virgin 1958). Thereafter the amount of chlorophyll *a* formed pro hour is around 0.03 milligrams per gram of fresh weight. The rate of formation is at the beginning independent of the light intensity as the process is determined by the formation of the precursor, protochlorophyll. The formation of this pigment is a slow process (Virgin 1955 b), while the transformation protochlorophyll \rightarrow chlorophyll *a* is a fast reaction (Smith and Benitez 1954).

By the aforementioned short light treatment followed by a dark period of five hours, the period of slow rate in the formation of chlorophyll *a*, "the lag phase", disappears or decreases.

During continuous irradiation the absolute difference in chlorophyll *a* concentration between two leaf samples, one of which has been pretreated as above will become maximal after about two hours. During further continuous irradiation, the difference will persist constantly for several hours,

Figure 3. The chlorophyll formation (mg/g of fresh weight) in dark grown leaves of wheat during two hours of continuous irradiation with 4000 lux. Prior to the continuous irradiation the leaves were irradiated for two minutes with 146 erg/cm² · sec of monochromatic light of different wave lengths followed by five hours of darkness. The figures for the chlorophyll formation denote the formation in excess of that obtained without any pretreatment.



but will become relatively smaller as the amount of chlorophyll *a* in the two samples is increasing at the same rate.

Figure 3 shows the amount of chlorophyll *a* in dark grown wheat leaves after two hours of irradiation with 4,000 lux of white light. Prior to this period of continuous light, the leaves were given a pretreatment consisting of a short irradiation with 1.75 mjoule/cm² (146 erg/cm² · sec during two minutes) of different wave lengths followed by a stay of five hours in darkness. The values presented in the Figure represent the amount of chlorophyll *a* which is formed in the leaves in excess of that formed during two hours of irradiation, but without any pretreatment. Each point in the Figure represent one measurement with 45 leaves in the sample holder. The values for the chlorophyll *a* contents of the leaves represent the contents of the leaves exposed to the irradiation through the hole in the sample holder. Due to the uneven distribution of pigments from top to the base in a grass leaf (cf. the values for barley, Virgin 1955 a), the values may differ from those which will be obtained when the pigment contents of the whole leaves are determined.

In Figure 3 it is seen that the response is mainly restricted to red light with a main peak at around 660 mμ. There are also indications of minor shoulders at around 540 mμ, 600 mμ, and 700 mμ. The last one being rather doubtful, however. On the whole the response curve tallies essentially with previously reported action spectra for other photomorphogenic phenomena (e.g. Parker *et al.* 1946, 1950, Borthwick *et al.* 1948, 1951, 1952 a, 1952 b, Piringer and Heinze 1954, Haupt 1959, Mohr 1956, 1959, Withrow *et al.*

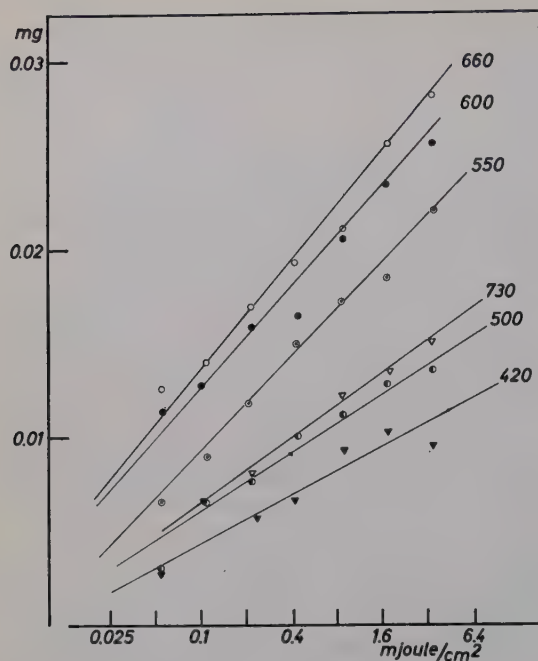


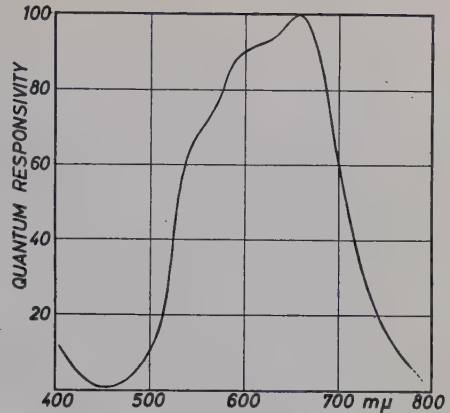
Figure 4. Regression lines of chlorophyll formation vs. incident energy. (Logarithmic scale).

1957). There is, however, a rather big response beyond 700 mμ, a fact which will be further dealt with in the discussion chapter.

The spectral curve in Figure 3 does not represent a true action spectrum as it involves equal incident energies instead of equal response. Also the logarithmic character of the response (see below) tends to reduce the relative magnitudes (cf. Withrow *et al.* 1957).

In order to check the degree of logarithmic response at those reactions within the wave length range applied, regression lines of induction of chlorophyll formation *vs.* log. incident energy were plotted for a few wave lengths (Figure 4). It is evident from this Figure that within the experimental error one can count with a logarithmic response. Outgoing from this it is possible to calculate a true action spectrum on a quantum basis. The calculations have mainly followed the directions given by Withrow *et al.* (1957). In Figure 3 it is shown that the lowest response within the wavelength range 400 mμ—700 mμ is at 450 mμ, which light under the prevailing conditions gives a response of 0.0055 mg of chlorophyll *a* per gram of fresh weight. From the regression lines for the different wave lengths which have been assumed to be linear, the energy was calculated which would be required to cause this response. The energy in mjoule/cm² was then converted to nano-einsteins/cm² according to the formula $n = \lambda E / Nhc$, where *n* is nano-

Figure 5. Action spectrum for the effect of light on the lag phase in chlorophyll synthesis based on the dotted curve in Figure 3. The ordinate shows the relative quantum responsivity calculated for a response of 0.0055 mg of chlorophyll *a* per gram of fresh weight per unit incident quanta. To convert to mg/g of fresh weight per nano-einstein/cm², the ordinant should be multiplied by the factor $5.8 \cdot 10^{-4}$.



einstein/cm²; λ is the wave length in mμ; N is Avogadro's number (6.03×10^{14} molecules/nano-mole; h is Planck's constant (6.63×10^{-31} mjoule · sec) and c is the speed of light (3.00×10^{17} mμ/sec).

From these calculations it is possible to obtain the quantum responsivity in milligrams of chlorophyll *a* per gram of fresh weight per nano-einstein per cm² based on a response of 0.0055 mg of chlorophyll *a* per gram of fresh weight. The result is seen in Figure 5. The values for this curve are based on the dotted line in Figure 3.

A fully correct calculation of an action spectrum based on quantum response implies a regression line for every single wave length as it is not certain that the linearity holds true down to zero energy for all wave lengths. At these calculations such a linearity has been assumed. The spread of the single determinations made it impossible to study an eventual divergence more closely. A smaller divergence in this respect will, however, only affect the spectrum calculated in minor details. The fact that in the photomorphogenetic response photoinactivation with far-red light has been shown to be proportional to energy, while the induction response is proportional to the log. of the energy (Withrow *et al.* 1957) is also of importance in this case. It should be emphasized, however, that the induction by far-red (730 mμ) at this present study seems to be proportional to the log. of the energy (Figure 4).

The action spectrum presented in Figure 5 is rather similar to that valid for the effect of light on the bean hook according to Withrow *et al.* 1957). The main difference is the stronger response in far-red.

2. The reversibility

A common feature for all red light induced photomorphogenetic phenomena hitherto investigated including photoperiodism and seed germination is that the reactions can be nullified by a treatment with far-red light

immediately following the red light impulse. The irradiation most active is that around 730 m μ . This reversible reaction has been interpreted as the result of a reversible change between two interconvertible pigments the red absorbing form of which is physiologically inactive and the far-red absorbing form the active agent at eliciting a physiological response (Hendricks *et al.* 1959 b). Recent studies indicate that the change in absorption elicited by light is due to a molecular rearrangement in the pigment molecule (Nakayama *et al.*, 1960).

In previous communications (Withrow *et al.* 1956, Wolff *et al.* 1957) a reversibility has been reported also for the light induced acceleration in chlorophyll formation but no figures are given. Enloe (1959) reports a small, hardly significant decrease in the effect of red light after a subsequent irradiation with far-red light. In no case a complete or nearly complete reversibility has been reported, comparable to that demonstrable in seed germination and in flower formation.

This problem whether far-red light can counteract the accelerating effect on chlorophyll *a* formation elicited by light of shorter wave lengths has in the present study been attacked in two different ways.

In a first series of experiments, the chlorophyll *a* formation has been followed during continuous irradiation with blue and red light without and with addition of far-red light. During irradiation without far-red light under the prevailing conditions the acceleration in the pigment formation sets in after about one to three hours (cf. Virgin 1958). The chlorophyll formation obtained in this way has then been compared with the rate of formation when the leaves, besides of being irradiated with blue and red light, are irradiated with strong doses of far-red light (730 m μ). Practically this could be done by irradiating the sample holder from two opposite sides.

A source of error as a consequence of this kind of arrangement is the formation of intensity gradients within the bundle of leaves. The opposite surface planes of the leaf bundle will become most strongly irradiated with more or less pure monochromatic light of the two kinds, while in the middle of the bundle, the relation between the energies of the two kinds will become quite different. These types of errors will to a smaller or greater extent always be actual at the irradiation of a living subject, where wave length depending light scattering and irregularities due to morphological inhomogeneities will be present. They will become particularly important here, however, where we have to count with opposite effects of the two light qualities. To what extent they will affect the result is difficult to predict.

In a second series of experiments the leaves were given short impulses of red and far-red light and five hours later continuous irradiation, as earlier described. The monochromatic red and far-red light was in these last mentioned series administered both after each other and simultaneously.

Far-red light alone does not transform protochlorophyll into chlorophyll *a* as protochlorophyll does not have any absorption beyond 700 m μ (Koski *et al.* 1951). Furthermore, the chlorophyll formation in continuous light, as far as the transformation is concerned, is independent of the intensity of the irradiation within a broad intensity range (Virgin 1955 b, 1958). This is due to the high light sensitivity of the protochlorophyll transformation. If far-red light has reversible effects on earlier stages in the chlorophyll formation, however, *i.e.*, on the mechanism for the protochlorophyll formation, which is accelerated by mainly red light, this should come out in the first series of experiments, where the light was administered continuously. Were it so that far-red light would completely reverse the accelerating effect elicited by blue and red light the curve for the formation of chlorophyll *a* should follow a straight line.

The results obtained do not speak in favour of any such influence of far-red irradiation, when administered continuously for longer periods of time together with red or blue light. Even if high energies of the far-red irradiation are used, an acceleration in the formation of the chlorophyll formation sets in after about the same time in all the experimental series. Small variations in the length of the lag phase can be found, it is true, but these variations do not statistically differ from the natural variation in the response of the material, which in itself is comparatively large, (*cf.* the spread of the points in Figure 3).

Evident effects, although small, of the far-red light was obtained, however, in the other series of irradiation experiments, whereby the monochromatic light was administered in short impulses followed by a dark period of five hours. After an irradiation for two hours with "white light" the chlorophyll contents were measured. The values presented in Tables 1 and 2 represent the chlorophyll formed in addition to that which will be obtained without a monochromatic pre-treatment (as in Figure 3).

From experiments where the impulses were given consecutively (Table 1) it is evident that a small reversibility between red light (660 m μ) and far-red light (730 m μ) exists, but the effect of red light can never be completely nullified by irradiation by far-red light. The cause for this is probably the fact that the far-red light in itself has an effect additive to the red light effect, (see Table 2) *i.e.* the red light absorbing pigment has an absorption which goes into the far-red region, which was also shown by the action spectrum in Figure 5.

Table 1 shows that one gets a reversal of the red effect only if a certain relationship exists between the intensity of the two kinds of irradiations. The differences are small, however, and hardly significant — *cf.* the spread of the values in Figure 3. The tendency is that at an increase of the energy

Table 1. *The efficiency of far-red light in reversing red light effects.* The red and far-red light administered consecutively. Each value for the chlorophyll *a* contents is the mean of four determinations.

Red (660 mμ)			Far-red (730 mμ)			Temp. °C	Interval between R and FR min	Chloroph. mg/g fr. w.
Intensity erg/cm ² . sec	Energy mj/cm ²	Time sec	Intensity erg/cm ² . sec	Energy mj/cm ²	Time sec			
803	0.80	10	—	—	—	22	0	0.024
803	0.80	10	657	0.65	10	22	0	0.022
803	0.80	10	657	1.97	30	22	0	0.022
803	0.80	10	657	7.88	120	22	0	0.024
803	1.77	22	—	—	—	22	0	0.026
803	1.77	22	657	3.94	60	22	0	0.023
803	1.77	22	—	—	—	22	0	0.026
803	1.77	22	657	3.94	60	22	5	0.026
803	1.77	22	657	3.94	60	22	15	0.026
803	1.77	22	730	4.38	60	22	0	0.022
803	1.77	22	730	4.38	60	22	5	0.027
146	1.75	120	—	—	—	22	0	0.028
146	1.75	120	256	3.07	120	22	0	0.022
803	1.77	22	—	—	—	0	0	0.024
803	1.77	22	730	4.38	60	0	0	0.022
803	1.77	22	730	4.38	60	0	5	0.020

Table 2. *The efficiency of far-red light in reversing red light effects.* The red and far-red light administered simultaneously. Temp. 22°C. Otherwise as in Table 1.

Red (600 — 690 mμ)			Far-red (730 mμ)			Chloroph. mg/g fr. w.
Intensity erg/cm ² . sec	Energy mj/cm ²	Time sec	Intensity erg/cm ² . sec	Energy mj/cm ²	Time sec	
292	3.51	120	—	—	—	0.026
292	3.51	120	496	5.96	120	0.023
—	—	—	496	5.96	120	0.013
73	0.88	120	—	—	—	0.023
73	0.88	120	496	5.96	120	0.021
—	—	—	496	5.96	120	0.012
73	0.44	60	—	—	—	0.019
73	0.44	60	496	2.98	60	0.019
—	—	—	496	2.98	60	0.011
73	0.22	30	—	—	—	0.017
73	0.22	30	496	1.49	30	0.017
—	—	—	496	1.49	30	0.011
219	0.66	30	—	—	—	0.017
219	0.66	30	219	0.66	30	0.016
—	—	—	219	0.66	30	0.010
219	1.31	60	—	—	—	0.018
219	1.31	60	219	1.31	60	0.019
—	—	—	219	1.31	60	0.010

of the far-red impulse above a certain value in comparison to that of the red light impulse, the resulting chlorophyll formation shows that the reversal is also decreased. An interval between the red and far-red treatment decreases the reversal, *i.e.* causes a greater additive effect of the two kinds of irradiations.

When the irradiations are performed at 0°C, a small reversal can also be shown to take place. An interval between red and far-red light does not here affect the reversal, which shows that the reaction, whereby the far-red absorbing pigment elicits its action on the protochlorophyll forming system is temperature dependent, which is not the case with the photochemical pigment conversion (Hendricks 1959).

In all experiments reported in Table 1, the far-red light was administered after the end of the red light impulse. In another series of experiments, the two light qualities were administered simultaneously. The red light source consisted of light within the region 600—690 mμ isolated from a beam of incandescent light by means of filters, while the far-red light came from the monochromator. Results from this series of experiments are reported in Table 2. That far-red light has an effect can be seen also here. If taking into account the effect of far-red light alone, the reversal gets still more obvious. It has never been possible, however, completely to reverse the effect of the red light.

Even if these last experiments concerning the effect of far-red light are not quite conclusive due to the energy gradient in the irradiation chamber, they do at least show that the reversible system is poorly developed in this red light response.

Discussion

From the action spectrum for the inhibition of the lag phase in chlorophyll formation, presented in Figure 5, it is evident that this effect of light is governed by the same mechanism as that acting in the photomorphogenetic phenomena. The reversal by far-red light is, however, very faint and in some instances hard to demonstrate, at least for dark grown leaves of wheat as found in the present investigation. Energies of far-red light which in most other photomorphogenetic reactions elicit a strong reversal of the phenomenon previously initiated by red light has here sometimes even an opposite effect, *i.e.* works as red light. This fact is clearly shown by the action spectrum for the photoinduction which points to a response far in the far-red region.

Failure of photoreversible control of flowering has recently been reported by Nakayama *et al.* (1960) in *Pharbitis nil*. In this case the action spectrum

for the inhibition of flowering by light treatment near the middle of a 16-hour dark period shows a type of spectrum in principal similar to that shown in the present paper, *i.e.* with a response far in the far-red region.

Another fact, clearly demonstrable in the presented spectrum for the lag phase elimination is a comparatively high response in the region 500—600 m μ . A process with complete reversal in far-red has usually a lower response in this region (cf. Withrow *et al.* 1957). This high response is also shown by Pharbitis when the reversibility fails to take place (Nakayama *et al.* 1960). The latter authors have suggested three possible interpretations for this lack of reversibility, namely:

- a. The initial action effects its physiological stimulation before the attempted reversal.
- b. The red absorbing form of the pigment is not converted to far-red absorbing form by absorption of radiation.
- c. It is converted but this far-red absorbing form is not changed back to red absorbing form by absorption of far-red radiation.

From the experimental facts presented in this paper, it is evident that alternative *a* may hold true. A characteristic feature for this light-effect on the lag phase in chlorophyll formation is that the full effect of the light impulse can be seen as soon as after 4—6 hours. This in contrary to photomorphogenetic and photoperiodic phenomena, particularly flower formation, where the full effect cannot be seen until after one or several days. It seems therefore plausible to assume that the whole process of induction at this present pigment forming process goes much more rapid than in the other aforementioned phenomena. This would then account for the fact that even a short interval between the red and far-red impulse will decrease the effect of the far-red irradiation as was indicated in Table 1. If the temperature is kept low during the interval, the reversal will, however, become noticeable. As a reaction of red light is obtained, alternative *b* cannot be true, provided we still believe that the far-red absorbing form is the physiologically active one.

As far as alternative *c* is concerned, it can not be excluded that the small effect of far-red light at these phenomena is due to such a proposed inactiveness of the reversal mechanism.

The puzzling thing with the effect of far-red irradiation is that it no doubt has a small opposite effect to that of red light, but the prerequisite for far-red light to elicit its maximum reversal action has not been found. It cannot be excluded that an interaction may exist between a low energy reaction with a reversibility between red and far-red light in accordance with the photomorphogenetic phenomena first studied by Borthwick *et al.* 1952 a,

1952 b) and a high energy reaction whereby blue and far-red light plays the main rôle according to Hendricks *et al.* (1959 a) and Mohr and Wehrung (1960).

Summary

The action spectrum for the elimination of the lag phase in chlorophyll synthesis in wheat leaves shows a maximum at 660 m μ with minor shoulders at around 540 m μ , 600 m μ , and possibly at 700 m μ . A characteristic feature is the comparatively high response in the far-red region.

Disregarding the high response in the far-red, the spectrum tallies well with earlier published action spectra for photomorphogenetic phenomena.

The reversal mechanism is poorly developed. Small reversals of red light effects could be obtained for certain energy relations between red and far-red light, either administered consecutively or simultaneously. Due to great variations in the material it has not been possible to obtain fully quantitative values for the reversal. As far-red light in itself exerts a comparatively strong effect similar to that of red irradiation, the reversal only reaches a low percentage of the red-induced reaction.

The lack of reversal is discussed and compared with similar facts found in photoperiodic responses.

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Effect of Gibberellic Acid and 3-Indoleacetic Acid on Respiration of Onion Roots and Seedlings

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Numerous investigations have established the fact that the gibberellins bring about various growth responses (*e.g.* elongation, cell division, leaf expansion, etc.) in plants. The gibberellins have also proved to be useful in the induction of flowering and fruiting, in breaking dormancy, and in seed treatment to facilitate germination (17, 19).

Some attention has been given to the effect of gibberellins on root growth. In short term experiments gibberellins do not inhibit root growth, although in longer experiments there is general agreement that underground root growth is reduced in length, weight, and number (17). The overall effect of gibberellins in long term experiments (3 weeks) when it stimulates the shoot and inhibits the roots is to cause a marked change in the ratio of shoot weight to root weight (17). Brian *et. al.* (4) observed no significant effect of gibberellic acid on cress roots, or on the growth of roots of cress seedlings. Burström (5) has recently examined the influence of iron and gibberellic acid on the light sensitivity of roots. In this paper he also reviews some of the literature on the effect of gibberellic acid on root growth.

Extensive studies on the effect of gibberellins on metabolism have not been made, however recently work has started to appear on the effect gibberellin exerts on various enzyme systems (2, 15, 16, 18).

Stowe and Yamaki (17) call attention to the fact that the effect of gibberellins on plant respiration has not been studied in detail; however Nielsen and Bergqvist (11) have noted the stimulation of respiration of barley, wheat, timothy, rape, and pea seeds. J. Kato (8) has observed a stimulation of

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respiration, elongation, and water uptake in pea stem sections treated with gibberellic acid. Wittwer *et. al.* (18) have reported an increase in oxygen consumption in gibberellin treated bean stems.

Based on the fact that gibberellic acid is, in many cases, a growth stimulating substance it seems possible that it might stimulate the respiratory rate; therefore, the purpose of the experiments reported here is to examine the effect of gibberellic acid on oxygen consumption of various segments of the onion root tip. Respiration of roots grown in gibberellic acid, and of onion seed germinated in gibberellic acid is also reported. Preliminary measurements of the oxygen consumption of root tips exposed to 3-indoleacetic acid are recorded.

Materials and Methods

Onion sets were sprouted at $25 \pm 1^\circ\text{C}$ and the experimental samples were collected in the manner previously described in detail (12, 13). When the roots were two days old, the apical segments (0—5 mm. or 5—15 mm., measuring from the root tip toward the bulb) were used for experimentation. Samples of 100 mg. (wet weight) were transferred to Warburg reaction vessels containing either 3 ml. of $1/15\text{ M}$ KH_2PO_4 at a pH of 4.5, or gibberellic acid-buffer, or indoleacetic acid-buffer mixtures.

Oxygen consumption was determined at 30°C by direct Warburg manometry. In all cases the measurements were made in an atmosphere of pure oxygen, since Berry and Norris (3) have previously shown that the oxygen content of air limits respiration of the apical segments of the onion root under these experimental conditions.

In some cases onion sets were sprouted in different concentrations of gibberellic acid. This was accomplished by adding the necessary amount of gibberellic acid directly to the nutrient medium. Subsequent oxygen uptake measurements were made in buffer-gibberellic acid mixtures.

Onion seeds were germinated in petri dishes on filter paper moistened with distilled water or an aqueous solution of gibberellic acid of the desired concentration. Samples of 300 mg. (wet weight) were employed and the oxygen consumption measurements were made in buffer, or buffer-gibberellic acid mixtures.

Concentrations of gibberellic acid, and indoleacetic acid are expressed in parts per million (ppm), while oxygen uptake values are recorded in cubic millimeters per unit wet weight of tissue per hour.

Results

Oxygen consumption of onion root tips exposed to various concentrations of gibberellic acid. It is evident from the first line of Table 1 that gibberellic acid in concentrations of from 1 to 200 ppm. has no effect upon oxygen consumption of the root apex.

Table 1. *Effect of different concentrations of gibberellic acid on oxygen consumption of apical segments of onion root.* O₂ uptake expressed in μ L/100 mg. tissue (wet wt.) and hr. Values reported are the averages of several measurements.

Line	Notes	Conc. of gibberellic acid (ppm)					
		Control	1	10	50	100	200
1	0—5 mm. segment	110	104	113	101	93	104
2	0—5 mm. segment (1 hr. pre-exposure period)	89	—	—	85	89	82
3	0—5 mm. segment (2 hr. pre-exposure period)	61	50	70	75	72	—
4	0—5 mm. segment (3 hr. pre-exposure period)	36	30	34	59	34	38
5	5—15 mm. segment	42	42	41	45	—	—
6	5—15 mm. segment (2 hr. pre-exposure period)	37	40	44	47	30	34
7	5—15 mm. segment (3 hr. pre-exposure period)	38	—	—	36	37	40

In the next series of experiments root tips (0—5 mm.) were severed and allowed to stand in buffer or buffer-gibberellic acid mixtures for 1, 2, or 3 hours prior to respiratory measurement. The results are shown in Table 1, lines 2, 3, and 4. It is apparent that when measurements are made on root tips some time after severing them that the respiratory rate is lower (see control column). This is undoubtedly the result of the depletion of available substrate within the root during the time elapsed (14). In general the presence of gibberellic acid does not alter significantly the rate of oxygen consumption, however the possibility of slight stimulation (particularly in the concentration of 50 ppm) is indicated.

Predicated upon the fact that gibberellins are known to induce hyperelongation of some plant cells, the next series of experiments were conducted on the 5—15 mm. segment of the root. An examination of lines 5, 6, and 7, Table 1, reveals essentially the same points for these more basal segments as shown by the root apex; *i.e.*, in general the presence of gibberellic acid does not influence significantly the rate of oxygen consumption, although the possibility of slight stimulation persists in concentration of 50 ppm. with a two hour pre-exposure period. The oxygen uptake per unit weight of the 5—15 mm. root segment is lower than that for the apical segment.

Oxygen consumption of roots grown in gibberellic acid. Onion sets were sprouted in the nutrient solution to which gibberellic acid had been added, and subsequent measurements of oxygen consumption were made in buffer-gibberellic acid mixtures. The results recorded in Table 2 do not reveal significant differences between the experimental samples and the control. No

Table 2. *Effect of growing roots for two days in different concentrations of gibberellic acid on oxygen consumption of the apical segment (0—5 mm.). O₂ uptake expressed in μ l./100 mg. tissue (wet wt.) and hr. Values reported are the averages of five measurements.*

Concentration of gibberellic acid ppm	Control	Sprouted in gibberellic acid
3	121	122
6	118	101
10	108	110

visible differences were apparent between the roots sprouted in nutrient solution, and those sprouted in nutrient solution containing gibberellic acid.

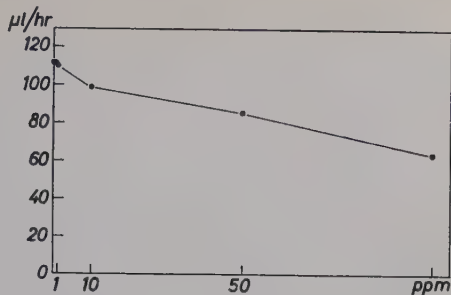
Respiration of onion seed germinating in various concentrations of gibberellic acid. Gibberellins are known to influence the germination and respiration of various seeds (11, 19), therefore in the series of experiments recorded in Table 3 onion seeds were germinated and subsequent oxygen consumption measurements were made in various concentrations of gibberellic acid. From an examination of the data it is apparent that exposure to concentrations of gibberellic acid of from 1 to 200 ppm has no significant effect on the respiration of the onion seedlings. The results yielded by a 96 hour exposure period appear to be out of line with the other data. Possibly some of the variation in respiratory rates may be accounted for by the fact that the roots of sprouted seeds varied in length from 1—20 mm. even with the same exposure time. No obvious visible difference was recognizable in the degree of germination shown by the seeds exposed to gibberellic acid when compared to the controls germinated in distilled water.

Oxygen consumption of onion root tips exposed to various concentrations of 3-Indoleacetic acid. Apical segments (0—5 mm.) of onion roots were suspended in buffer containing 3-Indoleacetic acid and oxygen uptake was

Table 3. *Effect of germinating onion seed in different concentrations of gibberellic acid on respiration. O₂ uptake expressed in μ l./300 mg. tissue (wet wt.) and hr.*

Germinating period hrs.	Concentration of gibberellic acid ppm					
	Control	1	10	50	100	200
24	49	40	34	—	41	36
48	271	259	271	258	259	251
72	310	278	260	315	311	300
96	90	89	129	133	133	105
120	212	145	142	136	172	169
144	159	126	165	168	131	190

Figure 1. Effect of different concentrations of 3-indoleacetic acid on oxygen consumption of apical segments of onion root (0—5 mm.) On the abscissa IAA in p.p.m. (last point 100 ppm), on the ordinate oxygen consumption $\mu\text{l./hr.}$ and 100 mg wet tissue. (Each point plotted is the average of 5 measurements.)



measured. It is evident from the data presented in Figure 1 that 3-Indoleacetic acid in concentrations of 10, 50, and 100 ppm significantly inhibits respiration of these segments.

Discussion

From the data presented it appears that exposure to concentrations of gibberellic acid ranging from 1 to 200 ppm does not significantly influence the oxygen consumption of either the apical (0—5 mm.) segment or the 5—15 mm. segment of the onion root tip. Pretreatment of the root tips with gibberellic acid for a period of 3 hours also revealed no significant effect on respiration, nor did sprouting the roots in various concentrations of gibberellic acid. Wittwer *et al.* (18) have reported a stimulation of oxygen consumption by bean stems upon treatment with gibberellic acid. J. Kato (8) studying pea stem sections from the third internode has noted that 10 ppm of gibberellic acid brings about a 15 % stimulation of respiration, a 50 to 75 % increase in elongation, and a 20 to 60 % increase in water uptake. When the stem sections were pretreated with gibberellin for 8 hours the increase in respiration only amounted to 20 %. Perhaps at this point it should be recalled that Leivonen (10) showed that gibberellin solutions varying from 20 to 200 ppm have no effect on the frequency of mitoses in *Narcissus* roots. The gibberellins did not cause any change in chromosomes and did not induce polyploidy; however, differentiated cells were induced to divide. In Leivonen's experiments the treatment period was from 4 hours to 11 days in length. Y. Kato (9) in studying the effect of gibberellins on root cells of the onion reported that concentrations of 100 ppm retard division of the root tips cells, however Leivonen (10) found that a concentration of 70 ppm did not prevent mitoses in onion root tips. Kato further noted that a solution as weak as 10 ppm somewhat inhibited growth of the roots. In the experiments reported here this effect was not noted.

Gibberellin is known to stimulate germination of various seeds. The promotion of lettuce seed germination has been extensively investigated by Kahn (7) as well as by Ikuma and Thimann (6). Gibberellin appears capable of assuming the role that red light plays in germination in all cases that were examined. Nielsen and Bergqvist (11) have reported that respiration of barley, wheat, timothy, rape, and pea seeds was stimulated by soaking the seeds in various concentrations of gibberellic acid (1 to 200 ppm) for 48 hours. Respiration was measured for 50 hours following soaking, by trapping the CO₂ produced. Seeds of barley and wheat showed the greatest stimulation while rape seeds gave a respiratory increase of only about 10 %. They suggest that the response is so consistent with barley seed that it may be useful as a biological assay method for low concentrations of gibberellic acid.

The data included in this report do not reveal any marked influence of gibberellic acid on germination or on respiration of onion seeds. It should be noted however that these experiments differ from those of Nielsen and Bergqvist in that we measured O₂ consumption for short time periods (1 hour) whereas they measured CO₂ production for some 50 hours following treatment.

The inhibition of oxygen consumption by apical segments of the onion root tip exposed to concentrations of 10, 50, and 100 ppm of 3-indoleacetic acid is in accord with the classical view of the auxin relations of roots with regard to growth. Åberg (1) points out, that in both intact and isolated roots, the auxin content is well over the optimum level, and normally only inhibitions of longitudinal growth result upon further auxin applications. He also calls attention to the fact that direct stimulation of the longitudinal growth of roots by low auxin concentrations has never been convincingly demonstrated.

Summary

The oxygen consumption of apical segments (0—5 mm. and 5—15 mm.) of the onion root tip exposed to gibberellic acid (in concentrations of 1 to 200 ppm) has been measured by direct Warburg manometry. No significant effect of the gibberellic acid on respiration could be detected in short term experiments.

A pre-exposure of the root segments to gibberellic acid for a period of three hours was also without effect on the oxygen consumption, as was sprouting the roots in gibberellic acid, or germinating onion seed in gibberellic acid.

An inhibition of oxygen consumption of the apical segment of the onion root is caused by 3-indoleacetic acid in concentrations of 10, 50, and 100 ppm.

We wish to express our gratitude to Merck and Co., Incorporated, Rahway, New Jersey, for generously supplying the sample of gibberellic acid (potassium salt) used in this study.

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Production by *Taphrina deformans* of Substances Stimulating Cell Elongation and Division

By

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Introduction

Auxin production, early demonstrated in fungus cultures (19), was associated with high tryptophane levels in the media (27). An important source of this activity was shown to be indole-3-acetic acid (IAA). Numerous later studies have indicated that IAA is a common fungal metabolic product, with or without tryptophane added to the media (12).

Auxin production by the fungus has been suggested to be a prerequisite for the ability to incite hyperplastic and hypertrophic growth of plant neoplasms (2, 3, 18, 29). Gall tissue has, in fact, been shown to contain abnormally high auxin levels (12, 22). Shaw and Hawkins (26), however, showed that non-tumorous lesions may also contain high auxin levels. Furthermore, the work of Bitancourt (4) and Pilet (23) suggests that the high auxin levels of neoplasms are not due to microbial synthesis at all. Instead, the presence of the fungus may cause decreased IAA-oxidase activity, thereby permitting IAA to accumulate as a result of a low destruction rate.

Although auxin probably participates in gall development, the importance of fungal auxin production is in doubt. Furthermore, factors other than auxin may be involved (12). In this regard, callus and mycelial growth in cotyledon pieces infected by the rust *Melampsora lini* (Pers.) Lév. is stimulated by IAA only in the presence of coconut milk (28).

Several plant growth regulators have been demonstrated in higher plants. Some have been auxins, and others gibberellin-like (1, 14, 15, 21, 25). Although first demonstrated in cultures of the fungus *Gibberella fujikuroi*

(Saw.) Wr., gibberellin production is evidently not common among fungi (7). An isolate of *Aspergillus niger* van Tiegh. was shown by Curtis (8, 9) to produce a growth substance resembling neither IAA or the gibberellins.

Less common is the demonstration in cultures of fungi of substances that stimulate cell division. Brewers yeast has been shown to stimulate cell division in tissue cultures (17).

Taphrina deformans (Berk.) Tul incites neoplastic growth of leaves of the peach, *Prunus persica* (L.) Batsch. The production of IAA by this fungus has been demonstrated (6, 13). The present investigation was conducted to determine if this fungus produces other growth substances.

Materials and Methods

Stock cultures of *T. deformans* were originally isolated as ascospores from invaded peach leaves, and the colonies were stored on potato-dextrose agar (PDA) slants in a refrigerator. Transfers were made to fresh PDA slants at room temperature to produce inoculum (cells from colonies less than one week old). *G. fujikuroi* cultures were obtained from the American Type Culture Collection (A.T.C.C. No. 12617) and were maintained in a similar manner.

The substrate used in most cases was potato-dextrose broth without added tryptophane. This contained eight grams of potato extract (Difco) and 20 grams of dextrose in one liter of tap water. The pH was adjusted to seven with potassium hydroxide. Certain preliminary tests utilized Czapek's medium with and without added tryptophane. When without tryptophane, the sole source of nitrogen was nitrate.

One hundred ml of media were added to each 500-ml. Erlenmeyer flask, which was then autoclaved 20 minutes at 15 lbs pressure. The inoculated liquid cultures were constantly swirled on a rotary culture shaker providing an amplitude of about one-half inch at 160 cycles per minute. All cultures were incubated at $25 \pm 2^\circ\text{C}$. Except during brief handling periods, all cultures were maintained in constant darkness.

The contents of six culture flasks were extracted in preparation for paper chromatography. In a few cases, the extracting solvent was peroxide-free ethyl ether. In those cases the cultures were extracted three times in the dark at 0°C , five or more hours for each extraction. The first extraction was at the final pH of the media, the second was acidified with lactic acid, and the third was made basic with potassium hydroxide. The ether fractions were combined, evaporated under reduced pressure to a few ml., and were applied to chromatographic paper.

In most tests, extractions were with 95 % ethanol without pH adjustments. About 300 ml of ethanol were added to each of the six culture flasks, which were allowed to stand overnight in the dark at about 0°C . Fungus cells were removed by centrifugation and discarded, and the remaining liquid was rapidly evaporated to dryness in a rotary "flash evaporator", avoiding temperatures above 35°C . The dry residue in the evaporation flask was extracted for several hours with about 20 ml of absolute ethanol, after which the alcoholic solution was removed and concentrated to about 2 ml by evaporation in a stream of air.

Concentrated extracts were streaked with a tuberculin syringe on a line about 4 cm. from the bottom of Whatman 3MM chromatographic paper sheets, size 46 by 57 cm. The unidirectional chromatograms, without prior equilibration, were developed by the ascending method for about 24 hours in the dark at $25 \pm 2^\circ\text{C}$. Developing solvents were either isopropanol, ammonia (28 %), water, 10 : 1 : 1 (v/v), or 70 per cent ethanol. After drying, the developed chromatograms were examined by ultraviolet fluorescence. A four cm. vertical strip was then cut from the middle of the chromatogram for detection of the Salkowski reaction, using the reagent of Gordon and Weber (11). The remainder of the chromatogram between the starting line and the solvent front was cut into 10 equal transverse strips. Each strip was folded and placed in a separate 100 \times 15-mm Petri dish containing 10 ml of two per cent sucrose in 0.01 M phosphate buffer at pH 6.5. A similar strip, obtained above the solvent front, was used for a control. Into each dish were placed pea sections for bioassay.

Chromatograms of kinetin, when developed by 70 per cent ethanol, were compared with chromatograms of fungal extracts that had produced a kinin like response in pea sections. The location of kinetin was determined by ultraviolet fluorescence and by the Dische color reaction (5). It was further studied by observing the physiological effect on pea sections by dividing the chromatogram into 10 transverse strips and a control as before. However, into each individual Petri dish was placed 10 ml of an IAA solution at a concentration of one $\mu\text{g/ml}$. The location of kinetin could be determined by the inhibition of IAA-induced elongation accompanied by an increase in section diameter.

Seed of the "Alaska" cultivar of *Pisum sativum* L. were soaked overnight in water and planted in unused vermiculite in 8 \times 11 \times 6- in polyethylene pans. Total darkness was maintained at all times except during handling or cutting operations, when a weak red light was used. After seven days, sections were cut with a tool holding parallel razor blades 8 mm apart. Internode sections immediately below the apical crook were selected. Such sections have been shown to contain tissues capable of responding to both auxins and gibberellins (24).

Fifteen or 20 pea sections were placed in each Petri dish containing a portion of the chromatogram. After 24 or 48 hours, section lengths were measured at 10 \times magnification on the stage of a dissecting microscope. The diameter of sections at their widest part was measured with a Starrett dial gauge graduated to 0.0005 in. In an alternative method, sometimes used, the sections were photographed. Measurements were made at a later date by projecting section images to three times actual size and measuring with a flexible plastic rule.

Used for a bioassay specific for gibberellins were Phinney's (20) dwarf-1 and dwarf-5 corn, *Zea mays* L. Seed was planted in a uniform potting mix of loam, sand, and vermiculite (1 : 1 : 1) in 12 \times 24 in. flats in the greenhouse. After seedling emergence the dominant normal plants were removed, leaving the recessive dwarfs. These were thinned to a one-inch space between seedlings in rows. On the seventh day after planting, about $\frac{1}{2}$ ml of the filtrate or extract to be tested was placed in the cup formed by the primary leaf. Treatments were repeated on the following two days. Seven days after the initial treatment the length of the leaf base of the first and second leaves was measured.

Results

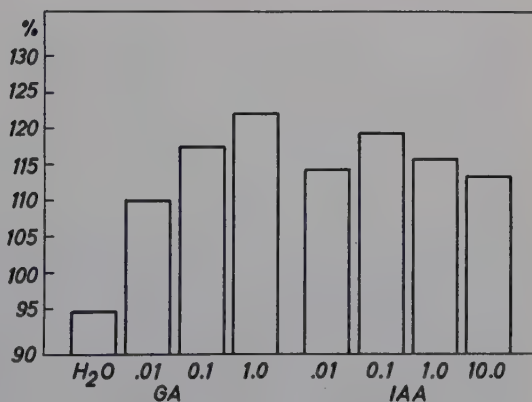
Experiments with commercial IAA and the potassium salt of gibberellic acid confirmed a response to each by etiolated pea sections consisting of 8 mm of internode tissue immediately below the apical crook (Figure 1). Though not particularly sensitive, in comparison with certain other bioassays, these substances were readily detectable in concentrations of 0.01 $\mu\text{g}/\text{ml}$. or less.

Preliminary tests with Czapek's medium showed very poor growth of *T. deformans* even with the addition of tryptophane. However, chromatograms of ether extracts of four-week-old cultures showed some pea section elongation in the IAA area. This was observed in extracts of tryptophane-free media as well as media enriched with tryptophane at one gram per liter. Nevertheless, the relatively slow growth in this medium necessitated the use of potato-dextrose broth in subsequent experiments.

In bioassays of chromatograms of ether extracts of cultures 1, 2, 4, and 5 weeks old, the single zone of extension was near the location expected for IAA when developed with either 70 per cent ethanol or isopropanol-ammonia-water. Judging by the extent of pea-section elongation, the auxin level was about as great at the end of one week as after longer periods. Color development with the Salkowski reagent generally resulted in the characteristic pink color at the expected IAA location. Several tests with ethanol extracts gave similar results.

If the cultures were held 4 to 6 months, the IAA level was frequently reduced. However, in ethanolic extracts of these cultures, a second zone of extension at R_f 0.2–0.4, was sometimes detected when 70 per cent ethanol was the developing solvent. No Salkowski color has been detected in the latter zone.

Figure 1. Pea-section elongation response (in % of sucrose control) in 24 hours to IAA and the potassium salt of gibberellic acid. Concentrations are micrograms per milliliter.



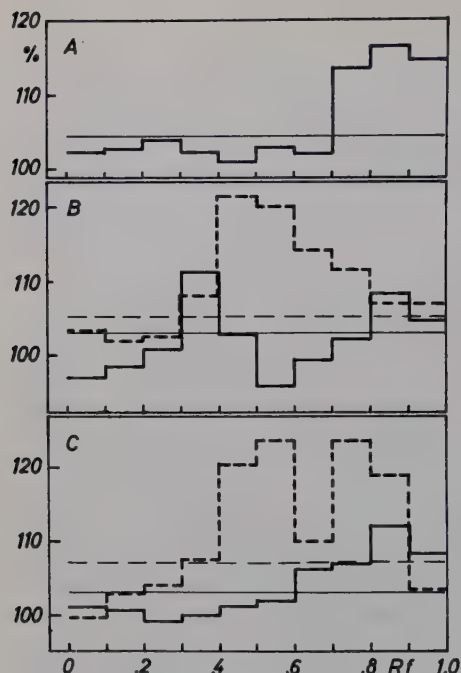


Figure 2. Pea-section elongation and lateral expansion in response to sections of chromatographs of ethanolic extracts of *T. deformans*. Solid lines represent elongation, and broken lines are section diameters, with both expressed as per cent of the final size of a two per cent sucrose control. The R_f of IAA when chromatographed in 70 per cent ethanol is about 0.8. The upper limits of three times the standard error are indicated by solid horizontal lines for elongation and broken horizontal lines for diameters. A. One-week-old culture. B. & C. Five-month-old cultures.

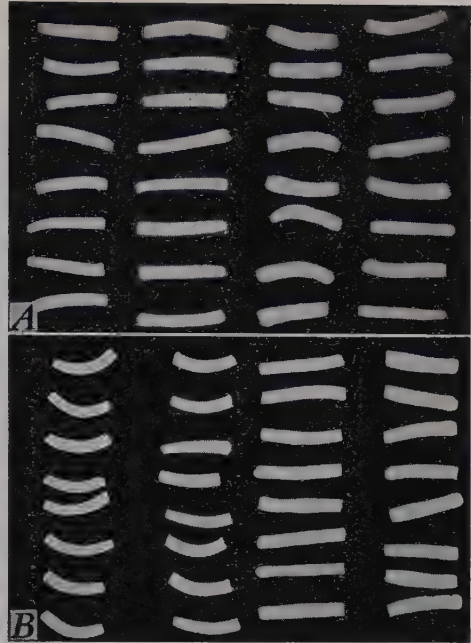
Acting on the possibility that the unknown zone of extension might be a gibberellin-like substance, dwarf-corn bioassays were conducted on two occasions. The first was by application of a filtrate from a five-month-old culture at final, $1/2$ dilution, or double final concentration of the culture liquid. However, no stimulation above water, or sterile media controls occurred although stimulation was observed with similar filtrates from *G. fujikuroi*.

Since dwarf-1 corn is reported insensitive to certain gibberellins, the test was repeated with dwarf-5 corn. To obtain greater concentration, the contents of six five-month-old culture flasks were extracted with ethanol and evaporated to a few ml in a "flash evaporator". The concentrated extract, however, again failed to produce a gibberellin response, whereas a similar extract of *G. fujikuroi* did.

Between the two zones of elongation described above there was a zone in which elongation was often inhibited, with a striking diameter increase observed sometimes. At other times, the diameter increase was detectable only by careful measurement or did not occur (Figure 2, 3 A).

In response to IAA or the unknown zone of elongation, pea section diameters increased when sections lengthened. This increase can be readily

Figure 3. A: *Pea-section response to areas of a chromatograph of an ethanolic extract of a five-month-old culture of T. deformans*. The chromatographic solvent was 70 per cent ethanol. Left to right, two per cent sucrose control; elongation in response to an unknown stimulatory substance at Rf 0.3–0.4; diameter increase, but elongation inhibition, between Rf 0.4 and 0.8; elongation at the IAA location, Rf 0.8–0.9. About $0.9\times$ magnification. B: *Response of pea sections to IAA and kinetin in two per cent sucrose*. Left to right, two per cent sucrose control; no IAA but 1.0 $\mu\text{g/ml}$. kinetin; IAA at 1.0 $\mu\text{g/ml}$. but no kinetin; and, IAA and kinetin at 1.0 $\mu\text{g/ml}$. combination. About $0.8\times$ magnification.



explained by cell enlargement. However, increased diameter enlargement accompanied by little or no elongation or actual inhibition suggested that cell division caused the diameter increase. Preliminary histological examinations of such sections indicated that cell division had been stimulated.

The diameter increase of the pea sections was compared with the reaction obtained with a known cell-division-stimulating substance, kinetin (6-furfurylaminopurine). Determination was made of the response of the pea sections to kinetin at concentrations of 0.01, 0.1, and 1.0 $\mu\text{g/ml}$. and IAA at similar concentrations, along with various combinations of the two materials, all in two per cent sucrose. The results (Figure 3 B, 4) show that the pea sections responded very little to kinetin without IAA. In combination with IAA, kinetin was observed to reduce the elongation response to IAA. Both kinetin and IAA were required for a diameter response similar to that observed in chromatograms of extracts of *T. deformans*.

A test with IAA alone in two per cent sucrose showed a diameter increase at concentrations supraoptimal for elongation. Part of this increase is due to stimulation of cell division. The high concentrations required, and differences in Rf, suggest that the stimulation of cell division by *Taphrina* extracts is not due to IAA alone.

The similarity of response suggested that kinetin might be the stimulant

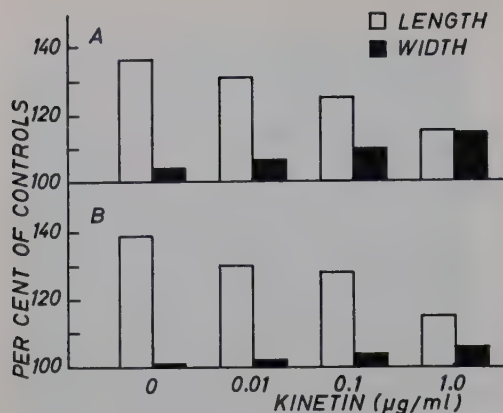


Figure 4. Pea-section elongation and lateral expansion after 24 hours in response to combinations of IAA and Kinetin in two per cent sucrose. Data are expressed as per cent of the final size of the sucrose control. A: With 1.0 µg/ml. IAA. B: With 0.1 µg/ml. IAA.

of cell division observed in extracts of *T. deformans*. The location on the chromatogram was a broad area extending from about R_f 0.4 to 0.8, when the chromatogram was developed with 70 per cent ethanol, using the pea section bioassay. In addition, a broad band of ultraviolet fluorescence was observed in this area. However, under the same conditions chromatograms of kinetin were not identical as determined by ultraviolet fluorescence, Dische color and bioassay.

Discussion

It is evident that cultures of *T. deformans* may contain several substances that affect the growth of higher plant tissue. One zone of elongation is Salkowski-positive and located on the paper chromatograms at the IAA position, and is therefore believed to be IAA. The other zone of elongation has not given a positive Salkowski reaction, but the reason may be an insufficient amount. Consequently, the unknown zone does not necessarily contain a non-indole compound. Failure to evoke a dwarf-corn response suggests that no gibberellin-like substances are present.

The stimulation of diameter increase with inhibition of elongation suggests that substances producing a kinin-like effect are present in detectable amounts in extracts of aged cultures. Whether the stimulation is the result of a single substance or the interaction of several has not been determined. Chromatographic evidence indicates that the substance is not identical to kinetin. No comparison was made with the stimulation of cell division by coconut milk or brewers yeast.

Only in combination with IAA does kinetin produce a striking pea-section response. In the case of chromatographed *Taphrina* extracts, a response

occurred without added IAA. However, when chromatographed with 70 per cent ethanol, IAA moves ahead of the zone of cell-division stimulation. Since some streaking of the IAA may occur, especially when crude extracts are chromatographed, it is possible that some IAA may have been present in the zone of pea-section diameter increase. Furthermore, the presence or absence of IAA may explain, in part, why a pea-section diameter increase sometimes did not occur even with extracts of aged cultures.

The diameter-stimulating substance (or substances) is possibly a metabolic product that accumulates with time. On the other hand, its presence may be associated only with cultures undergoing autolysis.

Although cell division was stimulated in pea sections having little or no elongation but a large diameter increase, it has not been determined that cell division is necessarily responsible for all the lateral expansion. Galston and co-workers (10) found that certain concentrations of benzimidazole inhibit the elongation of pea epicotyl sections while stimulating lateral expansion without increased cell division. Marinos (16) found that ethylene treatment of *Avena* coleoptile sections inhibited elongation but increased lateral expansion, with the lateral expansion dependent upon the supply of some factor from the endosperm. Consequently, the suggestion was made that while expansion usually involves growth in both the longitudinal and transverse directions, the direction of the expansion may be controlled by specific factors.

The significance of the different stimulating substances in relation to neoplastic growth appears worthy of investigation. The presence of high levels of IAA in such tissue is probably important in gall development but the importance of IAA synthesis by the parasite is in dispute. The production by the fungus of substances inhibiting IAA-oxidase activity might account for the elevated IAA levels found in galls. However, *T. deformans* incites leaf tissue to both increased cell division and cell enlargement and it is not certain this effect can be entirely explained by IAA. Consequently, the demonstration in this study of a cell division stimulating substance in culture suggests a similar stimulation might occur *en vivo* to contribute to gall formation.

Summary

Extracts of *Taphrina deformans* (Berk.) Tul., grown in potato-dextrose broth, were examined by paper chromatography for substances affecting the growth of higher plant tissue. Bioassay material used were eight ml internode sections taken immediately below the apical crook of seven-day-old etiolated seedlings of the "Alaska" pea, *Pisum sativum* L. Such sections respond to

indole-3-acetic acid (IAA), the gibberellins, and kinetin in the presence of IAA.

When ethanolic extracts were chromatographed with 70 per cent ethanol, a pronounced zone of elongation was nearly always observed at about the expected location of IAA. A second zone of elongation was sometimes detected. Dwarf-corn seedling bioassays indicated no gibberellin-like substances were present.

In a third zone, section elongation was inhibited while a large diameter expansion occurred. Histological examination suggests that all or a part of the lateral growth resulted from the stimulation of cell division.

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Volume Determination of Xylem Conduits in Stem and Petioles of *Phaseolus vulgaris* using Radiophosphorus

By

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Techniques are described in this paper for the volume measurement of xylem conduits which serve in the longitudinal transport of materials through plants. Such data may serve toward a better fundamental understanding of the plant transport network, since dimensions of this system, other than relative ones, are largely lacking. A more immediate and utilitarian objective, however, was the employment of values to compare sap composition with that of the external solution. Details of this phase of work appear in a later paper (3).

In the xylem volume studies given here, stem bases of test plants were first severed in a P-32 solution. Volume of solution drawn into the stem and petioles was determined by measuring sample radioactivity, and served as an estimate of xylem volume.

Materials and Methods

Bean seeds (*Phaseolus vulgaris*, variety Wade) were germinated and grown under controlled environmental and nutrient conditions according to techniques described elsewhere (2). Growth conditions were in brief: photoperiod, 16 hours; dark period, 8 hours; light intensity, approximately 1000 foot candles immediately above the plants; day temperature, 25°C; night temperature, 15 to 20°C; humidity, 50 %.

Plants were taken for study at about mid-point in the photoperiod when they were in a condition of high transpiration. Single plants were placed across the mouth of a mounted funnel in such a manner that the root-shoot junction was at

the base of the funnel. Root ends hung over one edge of the funnel, while the plant top protruded from the opposite edge. The funnel exit was sealed with a clamp.

Twenty-five ml of P-32 solution (as $\text{H}_3\text{P}^*\text{O}_4$) containing 8 ma P/l stable carrier (as KH_2PO_4) was added to the funnel, thus submerging the stem base. Exact activity of the isotope solution was previously determined by evaporating 50 lambda solution on a planchet and counting. An activity level in the vicinity of 25,000 cpm/ml was desired.

The submerged stem base was severed above the root-shoot junction with a sharp blade. The cut stem end was allowed to remain in the solution exactly 2 minutes after which time the leaves (minus petioles) and then the stem base (up to 2 cm above the solution meniscus) were removed. The remaining plant top was placed on a paper towel and sectioned into the following samples: P — petioles; E — epicotyl including the cotyledonary node and excluding the growing tip; HT — top half of the hypocotyl; HB — base half of the hypocotyl.

Fresh weights were recorded. Tissues were next cut into 2 cm sections and placed in aluminum planchets. Samples were dried at 120°C for 24 hours, and then brought to 450°C for 1 minute. The high temperature charred but did not gray-ash the material. Charring was preferred to gray-ashing because of reduced danger of sample spilling or air dispersion. Self absorption was negligible in either case, and therefore was not a serious consideration.

Samples were counted in a gas flow counter equipped with an ultra-thin end window ($150\text{ }\mu\text{g}/\text{cm}^2$). Counts were corrected for decay and background, but no correction was possible or believed necessary for geometry, self absorption, and backscatter.

Xylem volume was determined by the ratio solution to sample activity.

Results

Measurements of xylem volume derived according to the listed techniques are given in Table 1. Values were taken from a single group of 87 plants.

A striking feature of these data is the small indicated volume of the xylem. Xylem in individual samples measured between 0.003 and 0.007 ml, and totaled to only a little over 0.02 ml for all samples. Based on sample fresh

Table 1. *Xylem volume and other pertinent data for a single group of 87 plants.*

Sample	Xylem volume per sample ml	Sample fresh weight g.	Regression coefficient (ml volume/gram sample)	Coefficient of variation for volume-fresh weight values (%)
P	0.0030	0.324	0.0095	10.9
E	0.0069	0.385	0.0178	10.8
HT	0.0054	0.284	0.0190	8.2
HB	0.0060	0.382	0.0190	9.9
Total	0.0213	1.375		

weight, each gram of sample contained less than 0.02 ml xylem. Values may further be viewed on the basis of sample volume if reasonable assumption be made that specific gravity of test tissues approximated unity. Accordingly, each ml of sample contained less than 0.02 ml xylem. Thus, less than 2 percent of the sample volume may be considered xylem.

Of interest also is the fact that xylem volume for petioles was considerably less than for stem samples. Volumes for the 3 stem samples, on the other hand, were fairly similar. Fresh weight figures indicate that sample size could not have been responsible for the differences mentioned.

An indication of experimental error in test procedures is provided by the coefficients of variation which measured proximity of individual items around their mean. Coefficients ranged from 8.2 to 10.9 percent of the mean, depending on the particular sample. No effort was made to select plants for uniformity in this test, so that such figures probably represented extreme variation.

The procedures used to arrive at values in Table 1 evolved from a number of preliminary studies in which factors (other than xylem volume) which could possibly affect sample activity, were investigated. Of particular interest here was isotope which migrated laterally from the xylem and became resident in areas external to the conduit. Also of interest was isotope which became concentrated in the bound state by electrostatic adsorption. Inflation of test values could occur in each instance, and steps to avoid such error are described.

Lateral migration

A desired condition with respect to advancing test reliability was that all P-32 be confined to the xylem sap. Such a condition obviously does not exist in healthy plants, and radioions can be expected to migrate from the stream to, juxtaposed tissues. Definition between radioions in the stream and those located elsewhere in the sample was obvious for current purposes.

Time-course studies were undertaken to note if increases in sample radioactivity which occurred following filling of conduits with isotope solution could be clearly distinguished from the initial filling process. It was hoped that on the basis of such information, a plant-isotope exposure time could be selected which would allow satisfactory filling of xylem with isotope, but which would be too short to allow appreciable increase in sample activity by radioion deposition elsewhere.

Plants were treated in the standard fashion except that cut stem ends were allowed to remain in the isotope solution for periods of time ranging from 10 seconds to 72 minutes. Results are graphed in Figure 1. A sharp almost

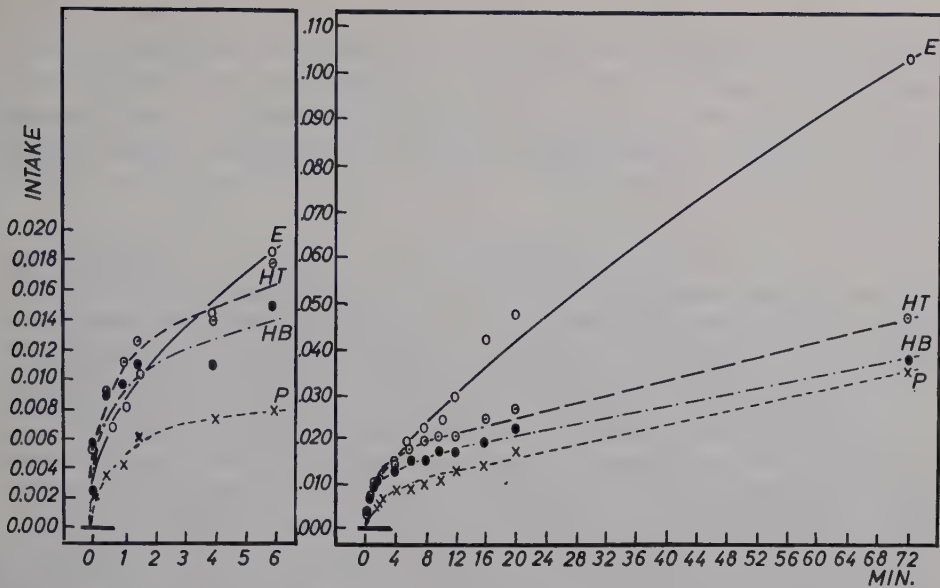


Figure 1. Time-course buildup of P-32 in stem and petiole samples. Each point on the curve represents the mean of 6 plants. The curve to the left is an enlargement of the initial 6 minute period. On the abscissa period of P-32 intake in mins., on the ordinate (cpm/g. fresh sample): (cpm/ml. solution).

vertical rise in activity of all samples occurred in the initial seconds of study, and tapered off to a more gradual increase with time. Transition between phases occurred at about the 2 minute mark.

The inference seems reasonable that rapid progressive filling of xylem conduits with isotope solution accounted for the initial sharp rise in activity, while subsequent deposition of isotope elsewhere accounted for the more gradual rise which followed. The 2 minute mark appears to represent that range of time where xylem filling with isotope solution is virtually completed, and where the second phase of buildup has not yet occurred to a serious level. The 2 minute mark, then, was selected as standard exposure time of cut stem to isotope solution in final procedures.

Electrostatic adsorption

In addition to the problem of lateral movement from the xylem, solute binding via electrostatic adsorption must also be considered as a source of error in test procedures. Included here are ions held by polar linkages to charged sites along the conduit walls. In this respect Charles (1) noted that

petiolar uptake of dyes (methylene blue, safranin) and antibiotics (streptomycin), all of which are plus charged, was very much slower than uptake of a negatively charged acid dye (fuchsin) and antibiotic (penicillin). Charles proposed that xylem surface was negatively charged and thus impeded uptake of cations to a much greater extent than anions. As a result of this report, the decision was followed in current studies to employ an anion form of tracer to measure xylem intake.

The matter of electrostatic binding of P-32 in current tests was pursued further. Techniques and interpretations in this area of work made recognition of the following: (a) That the adsorption reaction is inherently one of speed, and can be considered to have reached or approached an equilibrium state in the sample conduits by the 2 minute mark. (b) Adsorption of P-32 in the conduits can be expected to lead to an increase in sample activity and thus inflated volume values. This situation arises from the fact that under test conditions renewal of xylem contents occurs in short time intervals (see next section) so that adsorption depleted stream is rapidly replaced with fresh "full strength" solution. The level of P-32 in the sample stream at harvest time may therefore be considered similar in composition to the external solution. Adsorbed P-32 in effect adds to this level in the final counting process and boosts overall sample activity. (c) Electrostatic adsorption of P-32 is greatest in a carrier free system since every adsorbed phosphorus atom is potentially radioactive. As carrier is added, chances of a radioion participating in an adsorption reaction diminish. The adsorption effect on sample activity under current circumstances should therefore also diminish with carrier additions to the intake solution.

Experiments were undertaken in which xylem volumes of samples were determined according to accepted procedure except that intake solutions were employed which contained various levels of stable carrier (0.0, 2.0, 4.0, and 8.0 ma P/l). Results are given in Table 2. Carrier level did not affect results for P, HT, and HB samples, indicating little if any adsorption of P-32 here. In the case of E, however, the 0.0 P solution yielded significantly higher results than where stable phosphorus was added. It seems reasonable to attribute this difference to electrostatically bound P-32 in the carrier free sample.

Table 2. *Effect of stable phosphorus carrier in the intake solution on xylem volume values.*
Results are given as ml volume/gram fresh sample.

Sample	Intake solution carrier level (ma P/l)				
	0.0	2.0	4.0	8.0	LSD 0.05
P	0.0093	0.0092	0.0092	0.0092	0.0029
E	0.0322 ¹	0.0221	0.0197	0.0196	0.0057
HT	0.0237	0.0236	0.0234	0.0238	0.0030
HB	0.0183	0.0175	0.0172	0.0180	0.0027

¹ Significantly high at the 5 % confidence interval.

In summary, findings indicate that electrostatic binding of P-32 in P, HT, and HB samples was negligible. Significant binding occurred in the E sample, however, but could be reduced as a source of interference in xylem volume measurements by addition of stable carrier to the intake solution. A carrier level of 8.0 ma P/1 was finally accepted as standard procedure.

Other considerations

The relative importance of tension release and concurrent transpiration to xylem filling was studied by severing stems of some plants in growth solution, thus allowing stable material to surge into the system. Cut surfaces were kept in the stable solutions up to 6 minutes to ensure release of hydrostatic tension in the system, and were then transferred to a P-32 solution for 2 minutes to note extent of xylem intake following tension release. Samples were processed in the usual manner, and results compared with those from plants treated in the standard fashion where tension adjustment occurred in radioactive solution.

Results are given in Table 3, and show little difference whether P-32 intake occurred during or following tension adjustment. It appears that xylem filling in previous experiments was not primarily dependent on influx resulting from release of tension, but instead reflected transpiration which occurred concurrent with the period of study.

A further deduction is possible from Table 3; namely that passage of materials through the xylem occurred with considerable speed. In no instance was an activity gradient evident among samples, based on proximity of sample to isotope source. Apparently, P-32 which entered the xylem was transported through the system with such rapidity as to preclude detection of a gradient under test conditions.

Table 3. *Effect of hydrostatic tension release on test results.* Tension of some plants was released in stable solution and then moved to a P-32 solution, while tension of other plants was released in radiosolution according to standard procedures. Each treatment was replicated 6 times.

Time in stable solution before movement to P-32	ml volume/gram fresh sample			
	P	E	HT	HB
0 minutes	0.0068	0.0124	0.0129	0.0096
1 »	0.0070	0.0122	0.0122	0.0081
2 »	0.0082	0.0149	0.0123	0.0078
6 »	0.0070	0.0115	0.0114	0.0085
LSD 0.05	0.0018	0.0040	0.0032	0.0029

Additional aspects of techniques were studied including the effect on results of (a) location of cut, (b) cutting edge condition, (c) plant maturity, and (d) fluctuations in growth environment prior to plant utilization. Only brief mention is made of these factors to emphasize areas in which departures from accepted routines should be avoided.

Little difference in xylem intake of P-32 was noted whether the initial cut was made 2 cm above below, or at the root-shoot junction. Use of a dull instead of a sharp cutting edge yielded higher HB and lower E and P values. Xylem volumes were correlated in a positive and linear fashion with chronological age of plant over a 6 day period starting 3 days before treatment age. Changes in relative humidity over a range of 25 to 74 percent during the photoperiod just prior to the time the plant was studied had little effect on results. Finally, plants studied during the dark period yielded lower values for all samples than plants studied during a photoperiod.

Discussion

An attempt was made here to measure volume of xylem conduits which are functional in transport of materials through stem and petioles of young bean plants. Problems of lateral exit of isotope from the xylem as well as of electrostatic adsorption were considered, since these forces were believed to represent primary sources of error in test procedures. Rate of stream movement through the sample, as well as the relationship of tension and transpiration to xylem filling were also considered to the extent that such information aided in the explanation of test results.

Volume figures finally arrived at were reproducible within limits which were believed to be adequate as a working frame of reference in ion penetration studies considered later (3). Sight should not be lost, however, that these values are at best "apparent", and may well be susceptible to considerable refinement with more study. Particularly cogent for future consideration is the improvement of methods for isolating free isotope of the xylem stream from that fraction which is either bound or deposited elsewhere in the sample. It may be well in this respect to employ an isotope species which is non-functional in plant processes and which will not be subjected to metabolic concentration.

The balance of forces which can be suggested as contributing to error in current procedures tends toward an inflation of estimated volume values over true values. The effects of lateral movement from the xylem as well as of electrostatic binding within the conduits make for increases in sample activity, and thus a greater estimated volume. The elastic nature of the xylem wall itself may also be cited as a possible factor here. Relaxation of tension as the stem is severed could lead to a springing back of conduit walls and a resultant increase in respective volume.

According to the best information available to the author, these techniques represent a hitherto unexplored means of determining volume of functional xylem conduits. The results must be considered in this light and must stand review as further information regarding xylem dimensions becomes available. Whatever comparisons are made, the point should be remembered that current techniques do not appear to take into account conduits which are inoperative due to tyloses or other causes. Calibrations of xylem volume by visual means (as with microscope and camera lucida) may not, for instance, make such a distinction and may therefore differ from current figures.

Summary

An attempt was made to determine the volume of those xylem conduits which are functional in the passage of material through stem and petioles of bean plants. Test plants were grown to treatment size under controlled nutrient and environment conditions. Stem bases were then severed in a P-32 solution. Volume of solution drawn into the sample was determined, and served as an estimate of conduit volume.

Trial procedures were fashioned to eliminate as much as possible error resulting from lateral movement of isotope out of the xylem, as well as from electrostatic adsorption of isotope to conduit walls. Influences of lateral movement were largely avoided by limiting the time the plant was allowed to remain in the isotope solution. Electrostatic adsorption of P-32 was reduced by addition of stable carrier to the intake solution.

Xylem in individual samples measured between 0.003 and 0.007 ml, and totaled to a little over 0.02 ml for all samples. Less than 2 percent of any sample volume was attributed to functional xylem.

Evidence of rate of stream passage through the samples, as well as the importance of internal tension and concurrent transpiration to xylem filling were considered to the extent that such information aided in the explanation of test results.

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Evidence of a Barrier to Lateral Penetration of P-32 Across Roots of Intact Transpiring Plants, Based on Measurements of Xylem Stream Composition

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Ion movement from the root surface to final residence in the plant top can, in its most simplified form, be depicted as a sequence of three exents: (A) penetration across the root epidermis, cortex, endodermis, and parenchyma tissues to the xylem, (B) longitudinal transport in the xylem, and (C) metabolic deposition into ion sinks of the individual cell. Possibly the least understood of the three stages is that of penetration. The exact manner in which ions negotiate lateral transfer across the root to the xylem is still largely a source of conjecture in which metabolic and passive pathways are variously envisaged (reviewed by Broyer, 4, Epstein, 9, Kramer, 14, and others). A serious obstacle to more rapid progress in this area of inquiry is lack of detailed information regarding composition of the ingoing stream.

The bulk of information currently available regarding stream composition was derived in comparisons of xylem exudate from *decapitated* plants with composition of the ambient solution (reviewed by Bollard, 2). Results invariably show a higher solute content in the exudate than in the solution, suggesting that lateral penetration occurs against a concentration gradient, and is thus energy driven. Application of these findings to explain penetration for intact, transpiring plants may have limitations, however, since the possibilities for mass flow afforded by the transpiration stream are necessarily destroyed by decapitation. Although the subject is controversial, sufficient evidence exists to suggest that such flow may influence passage of ions into the plant (Winneberger, 26, Hylmö, 10, 11, Kylin and Hylmö, 15, Butler, 5, Phillis and Mason, 18, Wright, 27, Mason and Maskell, 16).

What information is available regarding stream composition in *intact* plants is largely based on comparisons of solute to solvent utilization by the plant over a period of time (10, 11, 21). These techniques have the disadvantage that only net uptake is measured. The ramifications of a return of nutrients from the root to the medium cannot, therefore, be assessed. Also, time course fluctuations in stream composition are submerged within a single average value.

Current work was undertaken to determine in direct fashion the composition of the ingoing stream of intact plants, and thus the quantitative aspects of ion penetration. Time course buildup of root introduced P-32 was measured in stem and petiole samples. An attempt was made to differentiate isotope in the xylem sap from that metabolically accrued in the samples. Finally, xylem sap P-32 was compared quantitatively with isotope in the external solution to determine whether changes occurred in stream composition during penetration.

Materials and Methods

Bean plants (*Phaseolus vulgaris*, variety Wade) were cultured from seed and grown to treatment size under controlled environment and nutrient conditions according to methods described earlier (8). Plants were taken for treatment at approximate mid-point in the photoperiod when in a condition of high transpiration. Isotope solutions to which plants were exposed during treatment contained a complete compliment of plant essential elements and enough P-32 as $\text{H}_3\text{P}^*\text{O}_4$ to yield in the neighborhood of 20,000 cpm/ml. Treatment solution temperature was 24°C in all tests.

The harvest plan following treatment was similar to that described elsewhere (8) and involved separating the plant into petiole (P), epicotyl (E), hypocotyl top (HT), and hypocotyl base (HB) samples. Samples were weighed, dried, charred, and then counted on a gas flow counter. Other details of technique pertinent to a particular experiment are listed in the appropriate section. In all procedures, care was taken to prevent root injury which could provide a port of direct and unrestricted isotope entrance into the plant.

Results

Time course buildup

Plants of treatment size were removed from the growth solution and roots placed directly into 50 ml aerated P-32 solution. Roots were allowed to remain in the isotope solution for various lengths of time ranging from 0

to 20 minutes. Plant tops were then placed on a bed of dry ice and frozen to the root-shoot junction. Tissues were solidified in this manner to prevent redistribution of xylem contents when internal hydrostatic tension was released during sectioning. Care was taken during the freezing operation to prevent contamination by radioactive solution adhering to the roots. Frozen plants were finally sectioned into P, E, HT, and HB samples, and processed.

A number of time course studies of this type were run. Results in all cases yielded essentially the same plot as shown for the 24°C plants in Figure 2, and are, therefore, not presented separately. An abrupt rise in activity occurred between the 2 and 4 minute mark for all samples. Slope for E samples in the post 4 minute period was considerably steeper than that of the other samples.

Measurement of xylem sap P-32

Time-course buildup of P-32 in test samples can, for present purposes, be divided into various broad categories. First is isotope resident in the xylem stream. Second is isotope which has left the stream and has been metabolically accumulated within the sample. Isotope electrostatically bound to the conduit walls may be suggested as another fraction. (Earlier work showed such binding to be negligible under test conditions). Isotope dispersed from the stream to the apoplast of juxtaposed tissues may represent still another fraction.

Overall procedures called for the measurement of P-32 in the sample stream as an indicator of lateral penetration. Significant error in such a measurement was anticipated from the masking effect of the metabolically accrued fraction. Techniques were therefore developed to measure stream P-32 under conditions where metabolic accumulation in the sample was arrested by low sample temperature. Care was taken not to alter root or leaf temperature during the cooling process so that sap composition, as influenced by root uptake and transpiration flow, was not affected. Stem chilling itself was shown by Johnston (13) to have little effect on transpiration flow through the stem.

Equipment built for this phase of work consisted of a two-piece plastic canopy into which were placed the stem and petioles of test plants (Figure 1). The canopy was then sealed completely with masking tape except for the top opening and the air inlet. A stream of cold air from the exterior of the building was adjusted to $3^{\circ} \pm 1^{\circ}\text{C}$ in a glycol bath, and then passed through the canopy, chilling the enclosed samples. A slight depression (ca. 1°C) occurred in temperature surrounding the leaves and of the root medium during the cooling process.

In practice, plants were sealed in the canopies, roots placed in 50 ml old growth solution, and a continual stream of cold air passed through the system. After a

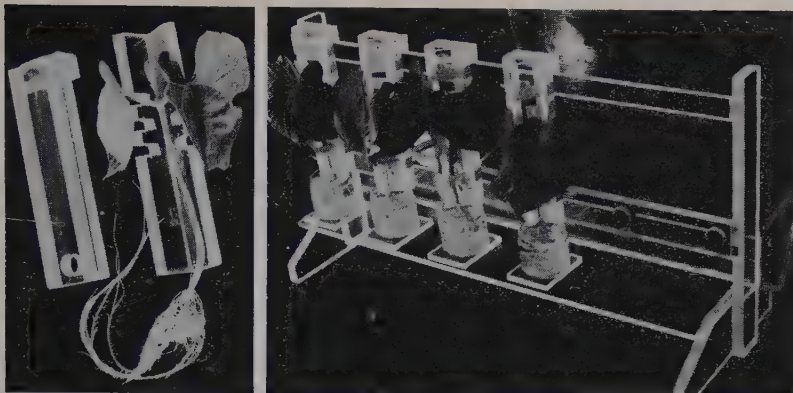


Figure 1. (Left) *Disassembled canopy used for cooling stem and petiole samples.* (Right) *Sealed canopies with plants.* Air inlet tubes lead from the glycol bath to the canopy base.

15 minute conditioning period, the old growth solution was decanted from around the roots and replaced with a 50 ml P-32 solution. Plant-canopy arrangements were moved to a bed of dry ice at specific time intervals following the isotope addition. Plants were solidified to prevent redistribution of xylem contents during sectioning. Samples were finally sectioned and processed. Check plants (24°C) were treated exactly as above, except cold air was not passed through the canopy.

Results of isotope buildup in 3° and 24°C samples are given in Figure 2. Sample temperature did not influence the initial phase of buildup which occurred between the 2 and 4 minute mark. Sample temperature did, however, affect buildup which occurred thereafter. Ever widening differences occurred between 3° and 24°C samples throughout the post 4 minute period. The influence of temperature was not absolute, however, and a depressed but nevertheless significant increase in P-32 occurred in 3°C samples during the post 4 minute period.

The various P-32 fractions which can be identified from this work are marked S, M, and X in Figure 2. It seems reasonable to attribute the abrupt pre 4 minute rise in activity (S) to passage of the P-32 front through the samples and filling of the xylem lumina with isotope solution. Such buildup was not sensitive to sample temperature since it was controlled by forces residing elsewhere in the plant.

The M fraction was sensitive to sample temperature, and logically represents P-32 metabolically accumulated within the sample.

The X fraction represents buildup which was not sensitive to sample temperature, and which occurred after the xylem was filled with isotope solution. The identity of this fraction remains unknown, but may fall within para-

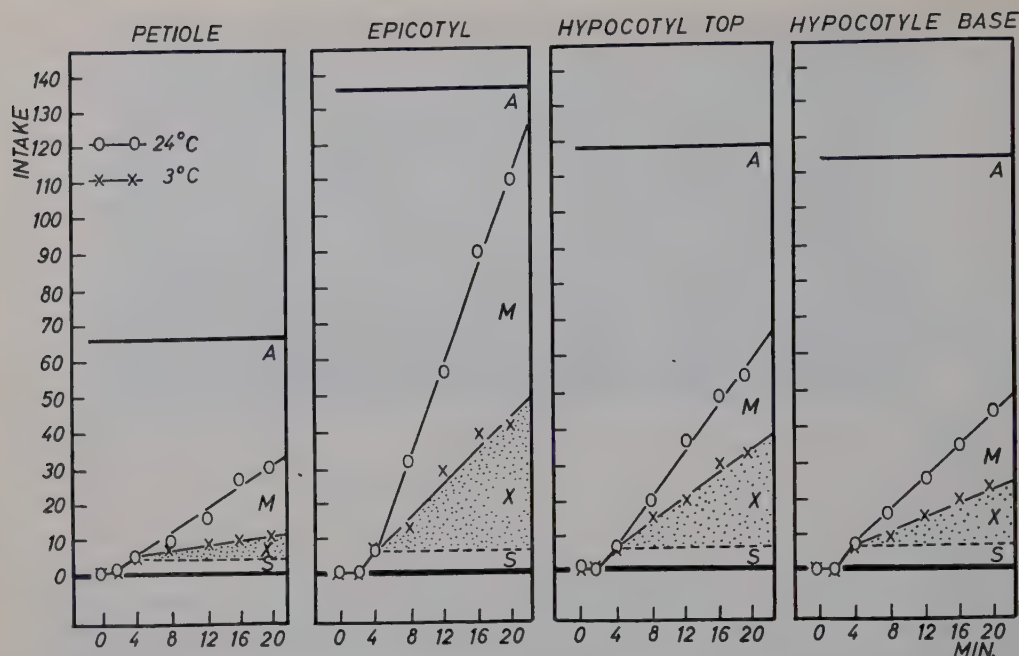


Figure 2. Buildup of root introduced $P-32$ in stem and petiole samples subjected to 3° and 24°C temperatures. "S" indicates activity due to initial passage of the isotope stream through the samples. "M" indicates metabolically accumulated isotope. The identity of the "X" fraction is unknown. A is the level of activity to be expected were the xylem conduits filled with unaltered solution. On the ordinate intake in $\frac{\text{cpm/g. sample}}{\text{cpm/ml. solution}} \times 10^{-4}$

meters set forth by one or a combination of the following proposals. First, the possibility exists that metabolic accumulation within samples was not arrested completely by the low temperature treatment. This proposal is supported by Butler (5), Broyer (3), Kylin and Hylmö (15), and Overstreet and Jacobson (17) who showed in their work that near zero degree temperatures did not necessarily arrest metabolic ion anabolism completely. The X fraction could also represent movement of isotope from the xylem stream to the apoplast of adjacent tissues. Physical movement of this type would not be eliminated by near freezing temperatures, and could therefore result in a gradual, temperature insensitive increase in sample activity. Finally, and most important from the standpoint of present considerations, the X fraction could result from an increased passage of isotope across the root, leading to a gradual enrichment of the xylem stream. Xylem stream composition, under such a situation, could not be considered time constant with respect to $P-32$.

Definition of xylem sap composition in the present experiment is complicated by this question of time constancy of sap P-32. Since the possibility exists that sap P-32 may increase in concentration as a function of time, it cannot safely be presented here as a single value. Instead, the necessity arises of defining a range within which the true values may lie. Sap P-32 following passage of the isotope front may therefore lie somewhere within the boundaries set forth by the low temperature samples in the post 4 minute period.

Xylem sap — ambient solution P-32 ratio

The primary purpose of this investigation was to study lateral penetration of P-32 across intact roots by comparing isotope in the xylem sap with that in the external medium. Before such a comparison can be made, additional information is necessary regarding the volume relations of the sap being considered. These data were acquired according to methods described elsewhere (8). In brief, stem bases of 12 transpiring plants were severed in a P-32 solution and the amount of isotope drawn into the stems and petioles served as an index of xylem volume and thus sap volume.

Once such volumes were established, calculations were made of activities which could be expected in the test samples in Figure 2, were xylem filled with unaltered solution. Results are given as A in the same figure, and show clearly that sap P-32 was considerably below the level contained in the root medium. Obviously, the bulk of P-32 was removed from the ingoing stream before it reached the test samples.

The quantitative features of the depletion process can be illustrated more clearly in Table 1 by the ratio of P-32 in the sap to that in the medium (Russell's "transpiration stream concentration factor", 20). Since xylem sap composition was assumed earlier to fall within a range, maximum and minimum ratio values are given. Results indicate that sample xylem contained somewhere between 0.05 and 0.33 of the isotope originally in the stream. It

Table 1. *Ratio of P-32 in xylem sap to that in the external medium.* Values refer to the time period following passage of the isotope front through the samples. Maximum and minimum ratio values are cited since sap P-32 was stipulated as falling within a range.

Ratio	P-32 xylem sap/P-32 root medium			
	Petiole	Epycotyl	Hypocotyl top	Hypocotyl base
Minimum ...	0.060	0.055	0.059	0.061
Maximum...	0.182	0.327	0.321	0.202

appears that stream depletion of P-32 occurred with considerable efficiency in the present experiment.

One further characteristic of the stream depletion process is worthy of mention. Evidence in all preliminary time course studies, as well as for experiments in Figure 2, indicated that the P-32 front passed through the samples 2 to 4 minutes following exposure of the root to isotope. Passage of the stream from root surface to sample can accordingly be considered to take not more than 4 minute. It follows that whatever changes occurred in stream composition prior to reaching the test samples, were limited to this short time period. The depletion process, then, as well as being efficient, apparently operated with considerable rapidity.

Discussion

The data show clearly that the level of P-32 in the xylem sap of test samples was only a fraction of that contained in the external solution. Obviously, the bulk of isotope was removed from the ingoing stream somewhere prior to reaching the samples. These findings are in general agreement with Hylmö (10), and Kylin and Hylmö (15).

The manner in which P-32 was removed from the ingoing stream is not clear. Functional depletion by root cells no doubt accounted for some of the isotope loss. However, the speed and efficiency with which the bulk of material was removed from the stream would seem to eliminate such a consideration as the primary process involved. The evidence instead suggests the existence of a specific barrier to transpiration passage of P-32 across roots.

Proposal of a barrier to solute passage across the roots of transpiring plants is in agreement with Kylin and Hylmö (15). The suggestion is made by these workers that the root acts as a sieve during passage of the nutrient solution moved by transpiration, and that the sieve action is not due to active uptake by root cells. Hylmö (10) more specifically postulated two separate pathways for entrance of water into the plant while ions have only one pathway. The medium drawn to the anticlinal cell walls of the epidermis is viewed as passing to the root interior without hinderance. On the other hand, the fraction which meets the vacuole of the epidermis is filtered of ions. Water passes through the tonoplast while the filtered ions in part diffuse back to the medium.

Russell *et al.* (20, 21) agree that a barrier to free lateral flow of solute exists, but that negotiation is primarily metabolic and against a free energy gradient. Considerable evidence of a sap medium ratio greater than unity

is cited in support of this view. It is of interest to note, however, that when experimental conditions approached those of the current tests (high transpiration, high phosphorus plants, high ambient phosphorus level), ratios for phosphorus less than unity (and in accordance with current findings) were reported.

The view of a functional epidermis screening ions from the transpiration stream is encouraged by Sandström (22). Removal of root epidermis by di-*n*-amylacetic acid allowed solution approximating the composition of the external medium to surge into the root. The endodermis has also been cited as a functional screen to ion penetration (Collander, 6, Scott, 23, Scott and Priestly, 24, Jacobson *et al.*, 12, Broyer, 4), but not without controversy (Prevot and Stewart, 19, Weibe and Kramer, 25).

The basic proposal of a barrier to transpiration flow of ions across roots can finally find support in findings regarding Hylmö's influx coefficient (*i*), and of root free space. Accordingly,

$$i = \frac{C_T}{C_M}$$

where C_M is solute to solvent ratio of the medium, while C_T is that ratio for the transpiration dependent stream. For a given C_M level, C_T and *i* are known to remain constant over a range of solution influx rates (10). To achieve such constancy, a greater number of ions must be filtered from the stream as passage of solution into the plant increases. It is reasonable to assume that at least a portion of the filtered ions back up in the root free space, and would add to any laboratory calculation of such space. Free space measurements in cases of high solution influx should, then, be of a higher magnitude than measurements for similar material where influx is reduced. Confirming evidence of such a relationship is indeed available. Bernstein and Nieman (1) reported an increased apparent free space (AFS) of pea and bean roots with increased transpiration. Extrapolations indicated that as much as a doubling in solute concentration of the space could theoretically occur due to transpiration. Kylin and Hylmö (15) also noted a trend toward higher AFS values in intact wheat with increased transpiration. Finally, AFS of truncated roots in which transpiration flow was destroyed was definitely lower than AFS for intact plants.

Summary

Quantitative aspects of lateral penetration of P-32 across roots of intact transpiring bean plants were investigated by measuring isotope which reached the xylem stream. Plants were grown to treatment size under con-

trolled environment and nutrient conditions, and were then placed in a treatment solution containing P-32. Isotope which appeared in petiole, epicotyl, and hypocotyl samples over a 20 minute period was measured. Xylem stream P-32 was at least partially separated from the metabolic fraction by subjecting only the sample to low temperature.

Time course buildup of P-32 in test samples following a distinct two-phase pattern. The first phase was an abrupt rise in sample activity 2 to 4 minutes following root introduction of isotope. This rise was not sensitive to sample temperature, and was attributed to initial passage of the isotope front through the samples.

Buildup which occurred in the post 4 minute period was in part sensitive to sample temperature. Reductions which occurred as a result of 3°C sample temperature were believed to represent isotope metabolically accumulated within the sample. Remaining activity was attributed to isotope in the xylem stream and to an unknown "X" fraction.

Xylem volume of test samples was determined. Calculations were then made of activities which could be expected in the time course studies had composition of the ingoing stream not changed with respect to P-32. Results indicated that the bulk of P-32 was removed from the ingoing stream before reaching the test samples. The rapidity and efficiency with which the isotope was removed from the stream indicated that functional depletion was not the primary process involved. Suggestion was made of a specific barrier to transpiration passage of P-32 across the root.

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Interaction of Indoleacetic Acid Oxidase, Phenol Content, and Gibberellic Acid in the Photoperiodically Controlled Growth of *Lupinus albus* L.

By

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Recent studies on the role of indoleacetic acid oxidase (auxin oxidase) in growth processes devote an increasing attention to the inhibitors of the enzyme. This work is stimulated by the discovery of the photoperiodic control of auxin oxidase activity in the long-day plants *Lupinus albus* (25, 8) and pea (6). It seems very probable that the inhibition of enzymatic breakdown of 3-indoleacetic acid (IAA) is to be explained by the stimulated biosynthesis of enzyme inhibitors in plants exposed to long days. It is generally accepted that the inhibitors of auxin oxidase are phenolic compounds (cf. 9). Clear-cut evidence for the presence and operation *in vivo* of phenolic compounds as indoleacetic acid oxidase inhibitors has been presented only for the pineapple enzyme (9).

The relation between the tissue level of phenolics and indoleacetic acid oxidase activity has not been studied so far. However, it has been pointed out that gibberellic acid (GA) enhances the level of auxin oxidase inhibitors (6, 7, 13, 28) exerting thereby an auxin sparing action. Therefore, a study on the interaction of auxin oxidase, phenol content and GA in the photoperiodically controlled growth of *Lupinus albus* was undertaken. It was shown that the content in phenolics and indoleacetic acid oxidase activity are in an inverse relation in *Lupinus albus*. GA did not influence the level of phenolics or the activity of auxin oxidase but in spite of this it stimulated the growth of the experimental plants.

Materials and Methods

Lupinus albus (var. "Gyulatanya édes") seeds were germinated for 4 days at 25°C in Petri-dishes on wet filter paper and were then transferred to pots. One part of the seedlings was exposed to short-day illumination (direct daylight for 8 hrs), the other part received long-day treatment (direct daylight for 8 hrs+illumination by incandescent bulbs with an intensity of 2000 lux at the level of plants, for 16 hrs). The treatment lasted generally for 2 weeks. In studies on the effect of GA seeds were germinated on filter paper moistened with 10 and 100 mg/l. GA-solutions respectively. After planting the growing point of each plant received an additional treatment with GA (0.5—0.5 µg. at four occasions in 2-day intervals). In an other series of experiments the plants were grown in nutrient solutions (Pfeffer) containing 10 and 100 mg/l. GA respectively. The results were essentially the same as those obtained with potted plants.

At an age of 14 days, water extracts were prepared from the first two leaves in a proportion of 3 : 1. After centrifugation the indoleacetic acid oxidase activity of the extracts was assayed manometrically (24) or by paper chromatography (8). Dialysis of the extracts and the measurements of enzyme activity after dialysis was carried out according to Stutz (24). Simultaneously the amount of total phenolics was determined in hot water extracts of the leaves (in samples corresponding to 10 mg. fresh weight) by the method of Swain and Hillis (26). Results are expressed in ferulic acid equivalents. In studies on the effect of GA the length of the hypocotyls, that of the first internodes, and of the petioles of the first 2 leaves was also measured. In the case of older plants leaves developed on the uppermost internode were used for the determinations.

Results

1. Content of phenolics and indoleacetic acid oxidase activity

As may be seen from Table 1, exposure to long days markedly promote the synthesis of phenolics in the first two leaves of 14- or 21-day old plants. Practically no auxin oxidase activity could be demonstrated in leaves of the same material (8). With the increasing age of the plants the phenol content of their leaves becomes higher. This increase in phenolics is correlated with a decrease of the ability of leaf extracts to decompose IAA if the plants are exposed to 8-hr photoperiods. After a while the enzyme activity, more or less suddenly, ceases. The critical phenol level might be around 30 µg. phenolics/mg. fresh weight (ferulic acid equivalents). If this limit is surpassed, the difference between the phenol content of leaves exposed to different photoperiods vanishes. This observation is remarkable, since at that time (one month old plants) appear the first floral primordia on long-day treated individuals.

Breakdown of IAA could not be observed in leaf extracts of 14-day old short-day treated plants, if the homogenates were mixed (1 : 1) with boiled

Table 1. *Total phenol content and indoleacetic acid oxidase activity in the leaves of lupine plants of different ages grown under 8-hr (SD) and 24-hr (LD) photoperiods.*

Age of plants in days	Total phenols as 10 ⁻¹ µg. ferulic acid in mg. dry matter		Auxin oxidase activity µl.O ₂ /mg dry matter			
			undialysed		dialysed	
	SD	LD	SD	LD	SD	LD
14	2.57 ± 0.25 ¹	3.23 ± 0.30	9.5	0 ²	4.9	4.1
21	3.00 ± 0.10	3.50 ± 0.15	8.2 ²	0	4.7	4.5
28	3.50 ± 0.12	3.58 ± 0.16	0	0	5.1	4.1
35	3.76 ± 0.10	3.78 ± 0.18	0	0	4.8	4.3
42	4.23 ± 0.14	4.00 ± 0.10	0	0	4.3	4.2
70	5.00 ± 0.16	5.15 ± 0.28	0	0	4.6	3.8

$$^1 \sigma = \sqrt{\frac{\sum m^2}{n-1}}$$

² In some cases O₂-consumption was observed after a lag period of 45 minutes.

extracts from individuals exposed to continuous illumination. The same effect was obtained with boiled leaf extracts of 28-day old or older short-day treated plants. Therefore, evidence was presented for the close correlation between phenol level and indoleacetic acid oxidase activity.

The objection could be raised against the above interpretation that in the leaves of older plants the auxin oxidase is probably broken down or inactivated. However, dialyzed extracts, in the presence of 2,4-dichlorophenol, exhibit a marked indoleacetic acid oxidase activity. Therefore, the enzyme is apparently present in the leaves of older plants as well, although in a latent (inhibited) state.

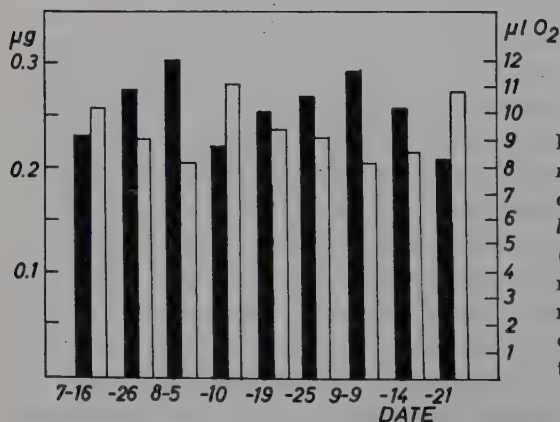


Figure 1. *Periodical changes in the phenol content and indoleacetic acid oxidase activity of the leaves of 14-day old lupine plants grown under short days (8 hr). On the left ordinate total phenols as µg ferulic acid in mg. dry matter, on the right ordinate auxin oxidase activity µl O₂/hr. mg. dry matter.*

The periodical change in phenol content and auxin oxidase activity in the leaves of 14-day old plants receiving 8-hr illumination is shown in Figure 1. It may be seen that the increase in phenol content is associated with a more or less concomitant decrease in auxin oxidase activity and vice versa. The variation of the figures obtained might be explained by changes in temperature. It is well established fact that the level of inhibitor(s) is greatly influenced by the temperature (8).

2. GA and indoleacetic acid oxidase activity

The effect of GA was assayed on 14-day old plants. The results are summarized in Table 2. Constant illumination — in accordance with our previous results (8) — greatly promoted the growth of the first internode. GA had the same effect on the hypocotyls on both daylengths, but the growth of the first internodes was stimulated by GA only in short-day treated plants. This is clearly visible in Figure 2. It is worthwhile to note that the petioles hardly respond to treatments with GA.

The increase of GA concentration from 10 to 100 mg/l. results in some additional elongation of the upperground organs, however, the differences are statistically not significant.

Table 2. *Effect of GA on the growth of 14-day old lupine plants under different photoperiods and on the dry matter accumulation, total phenol content, and indoleacetic acid oxidase activity their leaves.*

Photoperiod	GA mg./l.	Length in cm. of the			Total cm.	Growth related to the controls cm.	Dry matter in leaves %	Total phenols 10-1 µg. ferulic acid in mg. dry leaf matter	Auxin oxidase activity µl. O ₂ /l. hr in mg. dry leaf matter
		hypocotyls	1st internodiums	petioles					
SD	0	27.9 ± 4.6 ¹	2.7 ± 1.0	31.0 ± 6.6	61.6	—	9.96	2.57 ± 0.25	9.5
	10	46.6 ± 6.5	9.1 ± 2.9	34.7 ± 7.2	90.4	28.8	10.50	2.40 ± 0.30	9.3
	100	49.4 ± 8.9	11.8 ± 3.3	39.0 ± 5.8	100.2	38.6	10.62	2.62 ± 0.18	9.3
LD	0	52.1 ± 6.4	14.3 ± 3.8	40.7 ± 6.1	107.1	—	10.00	3.23 ± 0.30	0 ²
	10	73.6 ± 9.7	21.3 ± 6.4	41.9 ± 5.8	136.8	29.7	10.44	3.28 ± 0.35	0 ²
	100	74.8 ± 9.9	24.6 ± 7.5	48.0 ± 6.8	147.4	40.3	10.60	3.33 ± 0.25	0 ²

$$^1 \sigma = \sqrt{\frac{\sum m^2}{n-1}}$$

² In some cases O₂-consumption was observed after a lag period of 45 minutes.

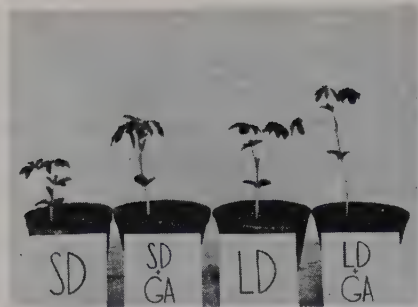


Figure 2. 14-day old lupine seedlings, treated with 10 mg./l. GA and untreated controls, grown on 8-hr (SD) and 24-hr (LD) photoperiods.

The results included in Table 2 show that plants exposed to short days respond to GA-treatment with more intensive growth than that of the long-day treated plants. This was also indicated by Wittwer and co-workers (29) with a number of plants. It can be deduced from the results that the effect of GA on plant growth is only an additive one. This statement is in agreement with recent results of Watanabe and Stutz (28).

It is known that the dry matter content of plants treated with GA is generally increased, the leaves exhibit chlorotic symptoms and the development of the root system is retarded (2). The dry matter content of the leaves of two-week old lupine plants was increased upon GA-treatment and the treated plants have shown the other two symptoms mentioned above.

Since the lupine seedlings exhibited several morphogenetical effects of GA-treatment, we expected to find a lowering of indoleacetic acid oxidase activity and enhancement of phenol level in the treated plants (6, 13, 28). However, contrary to our expectations neither the activity of auxin oxidase nor the concentration of phenolics was influenced by the GA-treatment, quite independently from the photoperiods applied.

Discussion

1. Content of phenolics and indoleacetic acid oxidase activity

Dawson and Wada (4) have shown that the content of chlorogenic acid in tobacco leaves is the highest in the summer months (long days). The stimulatory effect of light on polyphenol synthesis was also demonstrated by other workers (17, 27, 30). These statements hold true for lupine seedlings as well (Table 1.). As shown in Figure 1 there is an inverse relationship between the phenol content and auxin oxidase activity of lupine leaves. These findings support our earlier hypothesis (8) as to the involvement of the synthesis of phenolic inhibitors in the photoperiodic control of indoleacetic acid

oxidase activity in *Lupinus albus*. It is likely that during the ageing of the speed of inhibitor synthesis surpassed the synthesis of stimulators of the enzyme (23). This might explain the absence of enzymatic breakdown of indoleacetic acid in older plant individuals.

The phenolic inhibitors might play an indirect role in the regulation of the auxin level necessary for floral induction (15). This idea is supported by the observation that the phenol content of lupine leaves exposed to short or long days is different only until the floral primordia appear on the long-day treated individuals (Table 1.).

2. GA and indoleacetic acid oxidase activity

Treatment with GA promotes the growth of 14-day old lupine seedlings better under 8-hr photoperiods than under continuous illumination. Therefore, it can be assumed that in plants exposed to short days less endogenous gibberellin is synthesized than in those exposed to long days (1). Recent experiments of Harada and Nitsch (10) and Lang (14) indicated that the photoperiodic induction associated with the accumulation of gibberellin-like substances. It must be mentioned that the presence of gibberellin-like substances in *Lupinus* species has already been described (19).

Treatment of 2-week old lupine plants with GA results in stimulated growth similar to that induced by long-day treatment (Fig. 2.). In the GA-treated plants neither the activity of auxin oxidase nor the content of phenolics is changed (Table 2). Therefore, the stimulatory effect of GA can not be explained in our experiments by the direct (20—22) or indirect inhibition (due to phenolic inhibitors) of indoleacetic acid oxidase (7, 13, 28). The results of Kato and Katsumi (12) are similar in that they were also unable to observe the inhibition of auxin oxidase activity in etiolated pea seedlings treated with GA. It seems that the synthesis of a growth inhibitor is blocked by GA in young lupine plants (3) or the endogenous auxin level is augmented in the GA-treated individuals (5, 16, 18).

Watanabe and Stutz (28) found an inhibition of auxin oxidase activity in the growing points of 70-day old *Lupinus albus* plants treated with GA. However, in the leaves no indoleacetic acid oxidase activity could be demonstrated. On the basis of our results, this can be explained by the accumulation of inhibitors of the enzyme (Table 1.).

According to Galston (6) older pea plants contain less phenolic auxin oxidase inhibitors than the young ones. The results presented in this paper are opposite. Hillmann and Galston (11) have found a decreased activity of indoleacetic acid oxidase in pea plants exposed to red light. However, the auxin oxidase activity of *Lupinus albus* was not affected by the illumination.

All this taken together indicates that the operation of indoleacetic acid oxidase in *Lupinus albus* and in the pea plant respectively depends on different factors.

Summary

1. Leaves of 14- and 21-day old *Lupinus albus* plants contain a higher level of phenolics if exposed to 24-hr photoperiods than under short-day conditions. The above difference disappears from about the 4th week when the floral primordia on the induced plants become visible. The older is the plant the higher is the phenol content of the leaves.

2. Indoleacetic acid oxidase activity could be demonstrated only in the leaves of 14- and 21-day old plants exposed to short-day treatment. In the leaves of these plants the periodical fluctuations in phenol content are closely parallel with the changes in auxin oxidase activity.

3. The photoperiodic control of indoleacetic acid oxidase activity in *Lupinus albus* is mediated by a phenolic inhibitor the synthesis of which is affected by the photoperiodic treatment.

4. Treatment with GA promotes the growth of 14-day old lupine seedlings both under short- and long-day conditions, however, the activity of auxin oxidase and the level of phenolics in the treated plants is not affected.

5. The promotion of growth by GA in *Lupinus albus* can not be explained by the inhibition of auxin catabolism (auxin sparing action). Probably the enhancement of the endogenous auxin level is mediated by some other mechanism.

6. The role of the above processes in the photoperiodically induced growth of *Lupinus albus* is discussed.

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The Participation of the Red Far-Red Reaction System in Chlorophyll-Metabolism

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In recent years it has been repeatedly demonstrated that red illumination promotes, and far-red inhibits various light-dependent plant reactions, *i.e.* that a large number of photomorphogenic phenomena are controlled by the reversible red far-red reaction system (see review by Mohr 1960). The criterion for this system is that the effect induced with a low energy red dose can be annulled by a following far-red illumination.

In a brief communication Withrow *et al.* (1956) mentioned that a previous short light exposure to etiolated bean leaves causes an increase in the rate of chlorophyll formation when the plants is subsequently illuminated continuously. The most active spectral region causing this change in chlorophyll formation is at approximately 660 m μ , and this effect is nullified by post-irradiation with far-red at around 730 m μ . Virgin (1957, 1958) has further investigated this reaction. He has also found that the lag phase in the formation of chlorophyll a disappeared when prior to continuous white irradiation a pretreatment with a short red light impulse was given, followed by a five to six hours dark period. Virgins was further able to show that, with respect to the lag phase a red pre-irradiation treatment is about 22 time more effective than one with blue.

However neither Withrow *et al.* nor Virgin has published an exact reversibility curve. It, therefore, appeared of interest to study more closely the reversibility of this red induction on chlorophyll synthesis with far-red.

Physiol. Plant., 14, 1961

Material and Methods

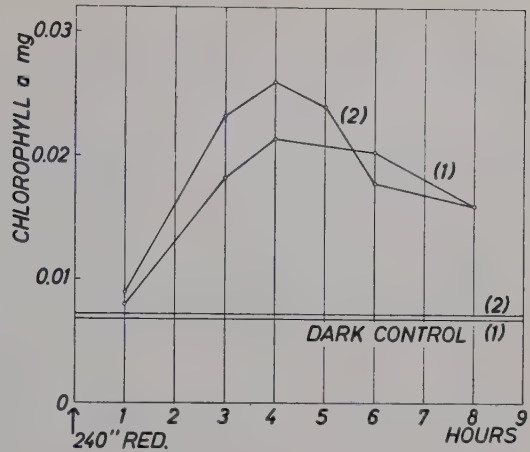
For this study seedlings of *Lepidium sativum* were used. Seeds were sown on three layers of 8×8 cm. white blotting paper (Charybdis, blotting-paper 9142/II), previously soaked for 5×10 hours in tap water, in colourless plastic refrigerator boxes $10 \times 10 \times 6$ cm. (trade-mark "Gerda"). Immediately prior to sowing all free water was poured off. 110 to 120 seeds were sown in 11 rows of 10 to 11 seeds per row. Immediately after 1 ml. of tap water was added to each of the four corners of the container (total 4 ml). At the end of one experiment free water was always present, hence the relative humidity within the containers remained high and constant for the duration of one experiment. A high humidity is necessary for a fast and homogenous release of the cotyledons from seed coats. Then the containers were wrapped in black plastic material and placed in a dark chamber at 25°C (see also Mohr 1959). After 48 hours all the cotyledons were still not freed from the seed coats; however, after 70 hours from sowing the capacity of the plant for chlorophyll formation had already decreased (Figure 1).

As a red source three Phillips TL 40 W/15 fluorescent tubes were used, which emit practically radiation only between 600 and 700 m μ . An Artlux reflector lamp (Phillips 500 W, 200 V) cooled by running water, served as the far-red source. Its radiation passed through 3 cm. of water and each of the Schott coloured glass filters RG₉ + BG₃ + KG₁ (each 2 mm. thick). With this combination practically radiation only between 700 and 900 m μ is obtained. In the following experiments the plants were placed at either of the two distances where the measured intensities were 7,050 erg/cm.² sec and 14,100 erg/cm.² sec respectively. For white light six fluorescent tubes (Osram) HNT : HNW 1 : 1 were used. Although the intensity varied between 3,000 and 3,200 lux, it remained constant for each individual experiment. For irradiation lasting more than 24 seconds the covers of the plant containers were replaced with colourless glass plates. When at the end of one experiment, the decapitation lasted for more than 15 minutes, the containers were placed in a refrigerator at $+2^{\circ}\text{C}$. The analyses were immediately carried out by the following procedure: 180 cotyledons were homogenised together with anhydrous Na₂SO₄, a pinch of CaCO₃ and ether in a Potter-type glass homogeniser (Braun, Melsungen, Germany). The extract was filtered with the aid of a water pump through a layer of anhydrous Na₂SO₄ on a fritted funnel directly into a 25 ml. volumetric flask. The residue was twice more homogenised with fresh ether and finally repeatedly washed in the funnel until filtrate and residue were colourless. The extract was made up to volume. All manipulations were carried out in weak green light, obtained from a green Osram fluorescent tube HNE 202/3 40 W. The extract was stored in the refrigerator until shortly before its optical density was measured at room temperature at 661 and 642.5 m μ with a Zeiss spectrophotometer. The extinction values were converted to milligramms or μg chlorophyll a (Smith and Benitez 1954).

Experimental Results

According to experimental results of Withrow *et al.* (1956) and Virgin (1957, 1958), the potential capacity of plants for chlorophyll synthesis following a short exposure to white or coloured light reaches a maximum 4 to 6

Figure 1. Time course for the capacity of chlorophyll synthesis following a short red light illumination. (1) plants 70 hours old. (2) plants 60 hours old. On the ordinate chlorophyll a in mg/90 plants.



hours after this exposure. In order to find this maximum for our material the following experiment was carried out: Plants, grown in darkness at 25°C for 60 to 70 hours following sowing, were given a 240 second exposure to red light. Then at various times between 0 and 7 hours darkness the plants were given 2 hours of white light. Figure 1 shows that the chlorophyll formed during this white light exposure reaches a maximum with a 3 hours dark period, or 4 hours after the red exposure when the middle of the white light treatment is considered. This is in agreement with Virgin (1958, fig. 7).

Therefore in all experiment to follow, the plants were given white light 3 to 5 hours after red induction. A saturation curve was now determined. For this purpose 60 hours seedlings were induced with the logarithmic series 2.4, 24, 240, 2,400 seconds of the red light and after 3 hours darkness were placed in white light for 2 hours. The results are shown in Figure 2. The highest effect is reached in approximately 4 minutes, after which the curve levels off. This is in agreement with Virgin's curve for protochlorophyll formation (1958, Figure 2). It was now attempted to annual with FR the effect of a 240 second red induction of chlorophyll synthesis. Plants 60 hours old which had been induced with 240 seconds R were exposed to FR in the same logarithmic time series of 2.4, 24, 240, 2,400 seconds (7,050 erg/cm.² sec.) The results are plotted in Figure 3 (solid curve). With as little as 240 seconds FR the red induction effect was reduced by 30 %. Although the further progress of the curve is less assured statistically, it appears to continue horizontally. In order to see whether the 30 % reversibility value could be further increased a higher intensity (14,100 erg/cm.² sec) was tried, *i.e.* the plants were placed closer to the FR source. However, nor with this intensity was greater reversibility obtained (Figure 4). In both experiments

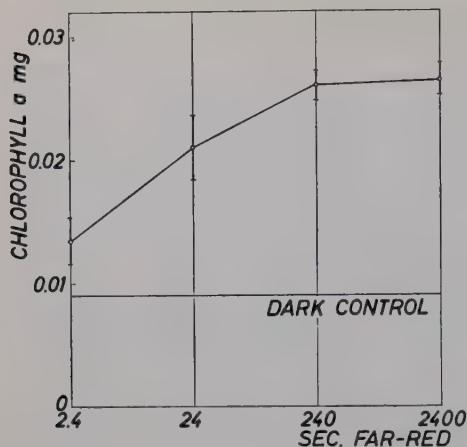


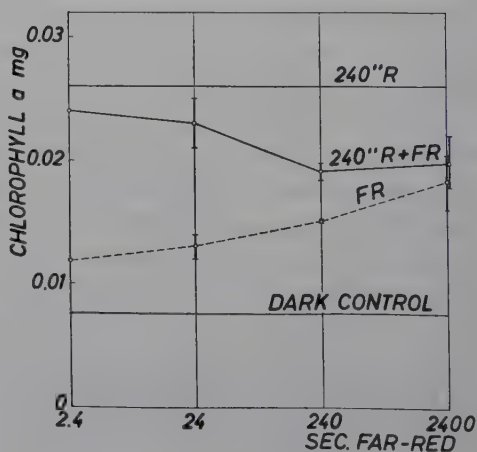
Figure 2. Dependence of chlorophyll synthesis on the energy time of red light illumination. The ordinate as Figure 1.

control plants were also given FR alone. The result is shown by the dashed curves (Figures 3 and 4) and we have seen, a certain increase in the capacity for chlorophyll synthesis was also obtained.

These two figures illustrate that the participation of red far-red reversible reaction system is certainly involved. In order to confirm the participation of the reversible red far-red system in chlorophyll metabolism a further experiment was carried out (Table 1). The results show a somewhat larger effect of red as well as far-red reversal than in Figure 2 to 4. It is seen that the last illumination determines whether induction or its reversal takes place, which is in full agreement with previous results on this reaction system. The higher value of the red effect following repeated red far-red treatments is

Figure 3. The reversibility of a 240 sec. red effect on chlorophyll synthesis by far-red illumination ($7,050 \text{ erg/cm}^2 \cdot \text{sec}$).

——— Reversibility curve.
 ---- Effect of far-red alone on chlorophyll synthesis. As Figure 2.



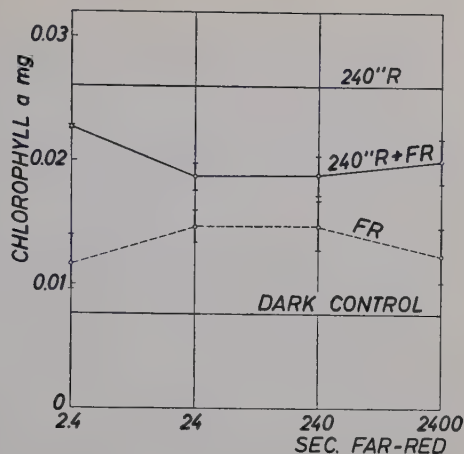


Figure 4. As Fig. 3 except that the far-red intensity was $14,100 \text{ erg/cm}^2 \cdot \text{sec.}$

Table 1. The induction of chlorophyll synthesis by red and reversibility with far-red several times repeated.

Illumination program (numerical values given in seconds)	Chlorop.a $\mu\text{g}/90 \text{ plants}$
240 R	25.9 ± 1.3
240 R + 240 FR	19.1 ± 0.68
240 R + 240 FR + 240 R	27.6 ± 0.74
240 R + 240 FR + 240 R + 240 FR	15.7 ± 0.59
240 R + 240 FR + 240 R + 240 FR + 240 R	28.9 ± 2.97

also in agreement with earlier findings (Borthwick *et al.* 1954, Mohr 1959) however these differences are not statistically significant. The differences between R + FR and R + FR + R + FR, which could not be easily interpreted, are not significant either.

Discussion

The results of the present work which further supplement the earlier data of Withrow *et al.* (1956) and Virgin (1957, 1958) clearly show that chlorophyll synthesis is promoted by a short exposure to red light (for our material and experimental set-up a somewhat greater than threefold increase as compared with the dark controls was obtained), and that this red effect can be lowered with far-red by approximately 30 %. If one repeats this red or far-red pretreatment it is clearly seen that the last given light quality is determining for the quantity of chlorophyll built.

From these results one could assume that chlorophyll synthesis is controlled by the same pigment system as other physiological or photomorphogenic processes seed germination, inhibition of internode growth in seedlings, the opening of the plumular hook of bean seedlings, chloroplast movement, influence on floral induction in long and short day plants, anthocyanin synthesis, *etc.*, see Mohr 1960. The peculiarity in the case of the photochemical formation of chlorophyll is that the lag phase for the synthesis is two hours and the maximum capacity for the photochemical synthesis of chlorophyll first appears 4 to 5 hours after the preliminary red induction. Of importance is the question whether the experimental results can be explained solely by the reversible red far-red pigment system or whether the participation of another component should be considered. From the view point of kinetics one could assume that the physiological active form P_{FR} , formed during red illumination, makes possible either the synthesis of a precursor of chlorophyll or the transformation of an already present precursor. The curve in Figure 1 of this paper or better, still, figure 1 of Virgin's paper (1957) shows great similarity to the curve for the time course of chlorophyll level and capacity of the plant for chlorophyll synthesis (Clauss 1954, Clauss and Rau 1956, Mitrakos *et al.* 1957, Clauss and Schwemmler 1959, Mitrakos 1959). If it could be shown that the first curve was a part of the second, then one would have to conclude that the red illumination also evokes the endogenous daily rhythm. Thus, above experimental findings provide the possibility to discuss the relation between endogenous daily rhythm (Bünning 1958, p. 41, 81, 88) and the red far-red reaction system. This, however, we wish to consider in a subsequent paper.

Summary

Chlorophyll synthesis, like other physiological processes, is controlled by red far-red pigment system: red promotes the synthesis whereas far-red acts antagonistically.

The author wishes to express his gratitude to Professor Erwing Bünning and Dr. W. Haupt of the Botanical Institute, University of Tübingen, for valuable advice during the investigations, and further to Mr. J. Landgraf who has revised the translation of the German manuscript. Address of the author: Botanical Institute, University of Thessaloniki, Greece.

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Scopoletin Glycosides in Tobacco Tissue

By

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The experimental regulation of the levels of a glycoside of scopoletin and the less abundant aglycone in tobacco callus and pith cultured *in vitro* has been demonstrated (Skoog and Montaldi 1961, Sargent and Skoog 1960) and the identity of the glycoside as scopolin reported (Sargent and Skoog 1960). Details of the identification of scopolin and the more or less complete characterization of three other glycosides of scopoletin present in tobacco tissue will be described.

Experimental

Material

Ethanolic extracts of both tobacco callus and root cortex (*Nicotiana tabacum* var. Wisconsin no. 38) when separately chromatographed on paper and viewed under ultraviolet light from a Mineralite SL 3660 hand lamp gave similar patterns of fluorescence. From this it was concluded that the fluorescent components of the whole tobacco plant qualitatively resembled those of isolated tissues cultured *in vitro*, and greenhouse grown material was used as a source of the substances in characterization studies. Tobacco plants about one meter tall, when examined under a u.v. lamp both before and after dissection of individual organs and tissues fluoresced most brilliantly in the cortex of the lower stem and primary root. After removal of the fibrous roots and soil particles adhering to the surface, the cortex of these parts of the plants was removed and quickly frozen at -15°C .

Extraction and purification

The frozen tissue was homogenized at room temperature in a Waring blender with sufficient 95 % ethanol to render the concentration of ethanol in the extract

80 % by volume. Solid material in the homogenate was removed by centrifugation and the supernatant evaporated to dryness under low pressure. The residue was dissolved in a minimum of 80 % ethanol and stored in darkness at 2°C. All steps in the extraction as well as subsequent purification and characterization procedures were carried out in darkness or weak diffuse light.

The blue-fluorescing components of the extract were separated chromatographically first with *n*-butanol : acetic acid : water = 4 : 1 : 2 v/v (BAW) and then with water. The extract was applied as a 51 cm. long streak to a number of 57 × 66 cm. sheets of Whatman 3 MM filter paper and these were developed descendingly with freshly prepared BAW. Scopoletin separated as a blue-fluorescing band at R_f 0.80—0.90 while a more brilliant band (F^2) was found at R_f 0.47—0.64. A number of less brilliant bands were also evident. The chromatogram was cut into the following sections:

R_f 0.29—0.38	II
R_f 0.38—0.47	III
R_f 0.47—0.64	F^2

Each band was eluted separately with water and rechromatographed with water as solvent. One or more bands were resolved on each of these chromatograms and the major one of each was cut out and eluted with water. With the exception of F^2 the eluates were separately rechromatographed with BAW and developed until the particular fluorescent band was close to the lower end of the paper. The paper was then dried and the band cut out and eluted with water. This eluate was evaporated to dryness and the residue dissolved in 80 % ethanol and stored at 2°C.

Preparation and analysis of hydrolysates

The substances were hydrolyzed by two methods, with hydrochloric acid and with an aqueous solution of emulsin. As it was desirable to examine intermediary as well as final products of hydrolysis, various concentrations of acid were used under standard conditions of temperature and time; whereas the enzymatic hydrolysis was done with a standard concentration of the enzyme, and the reaction was stopped after consecutive intervals of time.

A solution of the material to be hydrolyzed was evaporated to dryness in a 12 ml. tapered glass centrifuge tube, and, in the case of acid hydrolysis, the residue was dissolved in 0.2 ml. volumes of from 0.001 to 1.0 *M* hydrochloric acid. Each tube was capped with aluminum foil, autoclaved at 120°C for 20 minutes, and placed under vacuum for at least 90 minutes to remove all acid. The residue was dissolved in a minimum of 80 % ethanol. In the case of enzymatic hydrolysis 0.2 ml. of a 1.0 g/l. solution of emulsin was added to the glycoside in the centrifuge tube and incubated at 35°C for times ranging from 20 min to 9 hr. The reaction was stopped by the addition of 0.5 ml. 95 % ethanol followed by immersion of the tube in a steam bath to raise the temperature of the reaction mixture to boiling.

Constituents of the hydrolysate were separated chromatographically on Whatman No. 1 paper with BAW most frequently used as solvent. The entire content of one tube was applied as a single spot. After development of the chromatogram fluorescent spots were detected and photographed under the u.v. lamp. The film was protected from u.v. light by a Corning 3389-4308 filter attached to the camera. Reducing substances on the chromatogram were detected by the alkaline silver nitrate method

of Trevelyan, Proctor, and Harrison (1950), and the presence of reducing sugars from replicate hydrolysates was confirmed with benzidine and 3,5-dinitrosalicylate reagents (Block *et al.* 1952, p. 82).

Determinations of scopoletin and reducing sugar

Quantitative estimates of the amounts of scopoletin in aqueous eluates of chromatograms were made fluorometrically (see Sargent and Skoog 1960) and reducing sugars present in similar eluates were determined by means of modified Nelson's reagent (Somogyi 1952).

Melting point determinations

Melting point determinations were made by the conventional capillary tube method.

U.V. absorption spectra

U.V. absorption spectra of the substances in 80 % ethanol were obtained with either the Beckman D.U. or the Cary II spectrophotometer. Scopoletin and F² were obtained in milligram quantities and their absorption spectra were determined in the Beckman instrument. Only minute quantities of the other substances were purified. Prior to determination of their absorption spectra in the Cary spectrophotometer, each was chromatographed on Whatman No. 1 paper with acetone as solvent and eluted from the paper in water which was subsequently evaporated. Because of optically dense material originating from the paper itself, it was necessary to run a blank chromatogram and elute a control strip for use as a blank in the instrument.

Scopoletin standard

A pure sample of scopoletin had been prepared from an acid hydrolyzed extract of fibrous tobacco roots (Sargent and Skoog 1960). This crystallized from water as pale yellow needles melting at 204° C.

Results

F²

The F² eluate from the second (water) chromatogram was evaporated to dryness in a stream of warm air and redissolved in a minimum of 95 % ethanol. On stirring an excess of diethyl ether into the solution, a voluminous white precipitate formed. This was filtered, washed with a little ethanol, then with ether, and dried. In the many solvents tested (Table 1) this substance partitioned on paper chromatograms as a single spot. It melted sharply at 217°C.

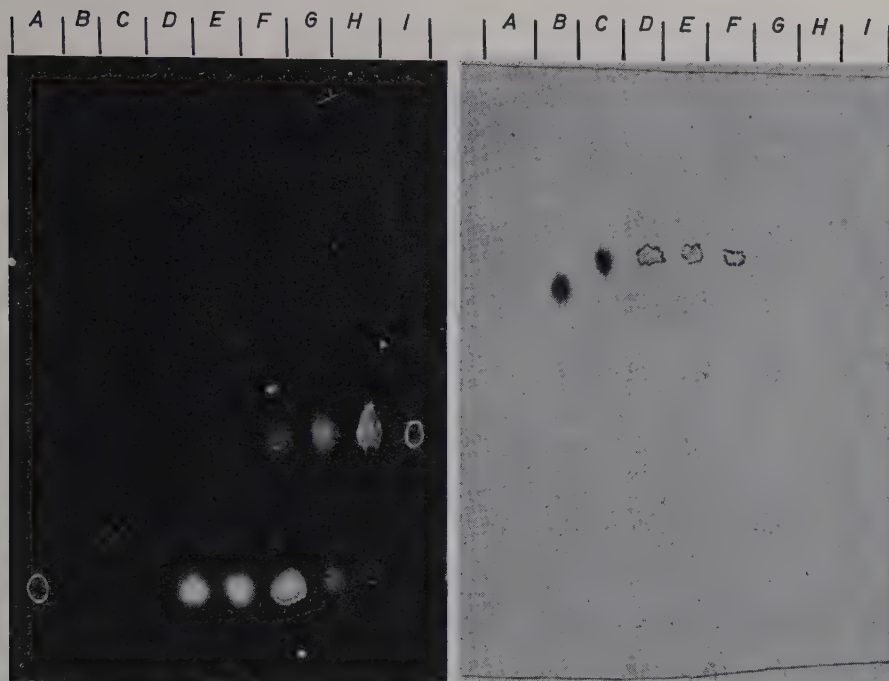


Figure 1. *Chromatogram of acid hydrolysates of F^2 . Left, photographed under u.v. light. Right, after alkaline silver nitrate treatment, photographed under white light. Solvent: BAW — descending. — A, scopoletin; B, fructose; C, glucose; D F^2 (HCl, 1.0 N), E, F^2 (HCl, 0.10 N); F, F^2 (HCl, 0.01 N); G, F^2 (HCl, 0.001 N); H, F^2 (water); I, F^2 .*

Hydrolysis of F^2 with increasing concentrations of acid and subsequent chromatographic separation of the products revealed the formation of a fluorescent substance travelling with scopoletin in BAW (Figure 1). The stronger the acid the more complete was the hydrolysis. Upon elution of the fluorescent product and rechromatography in other solvents it had R_f values identical with those of scopoletin. Also, its u.v. absorption spectrum matched perfectly that of scopoletin. It was concluded that the fluorescent product is indeed scopoletin.

Figure 1 also includes a photograph of the same chromatogram after treatment with alkaline silver nitrate. It shows that another product of the hydrolysis was a reducing substance separating with glucose on the chromatogram. Reactions with benzidine and 3,5-dinitrosalicylate confirmed the identification of this spot as a reducing sugar. Chromatographing this substance in a number of solvents failed to resolve it into more than one spot, and in all

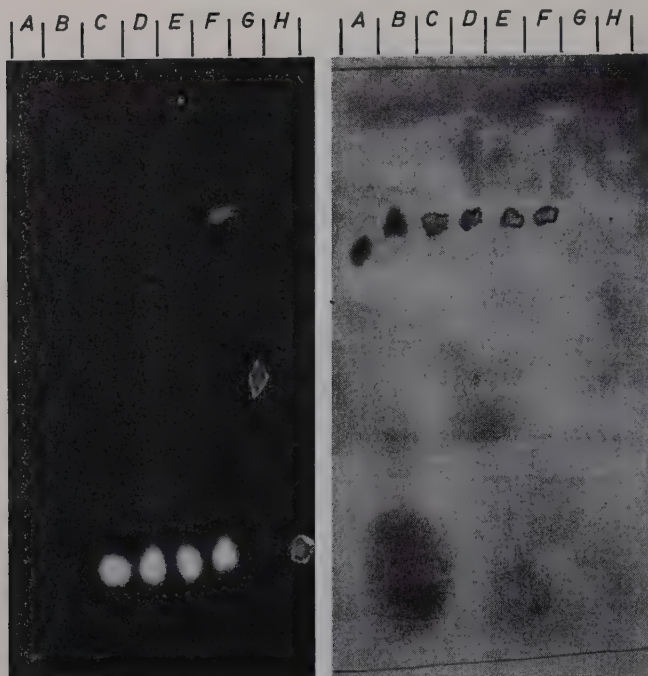


Figure 2. *Chromatogram of emulsin hydrolysates of F². Conditions same as for Figure 1. — A, fructose; B, glucose; C, F² (emulsin, 180 min); D, F² (emulsin, 60 min); E, F² (emulsin, 20 min); F, F² (emulsin, 30 sec); G, F²; H, scopoletin.*

cases it travelled with glucose. On this evidence it was concluded that this product is glucose.

Three quantitative determinations of yields in the hydrolysis of F² gave a mean scopoletin/glucose ratio of 11/10.

Hydrolysis of F² by emulsin was rapid (Figure 2). Even the few seconds in which emulsin was in contact with F² in the control [F² (Emulsin, < 30 sec.)] before the mixture was heated with ethanol was sufficient time for complete hydrolysis to scopoletin and glucose.

On the above evidence for the properties of F²; namely, that it yielded scopoletin and a single reducing sugar, glucose, by both acid and emulsin hydrolysis, that the molar ratio of these was 1/1, and that its m.p. was 217°C, it was concluded that F² is scopolin, the β -glucoside of scopoletin. This glycoside has been prepared by Merz (1932) and Chaudhury *et al.* (1948) and found by these authors to melt in the range 215—217°C. and 217—219°C. respectively.

II α

II α , the major constituent of section II, was subjected to acid hydrolysis in the same way as F² above. Again scopoletin was formed on complete

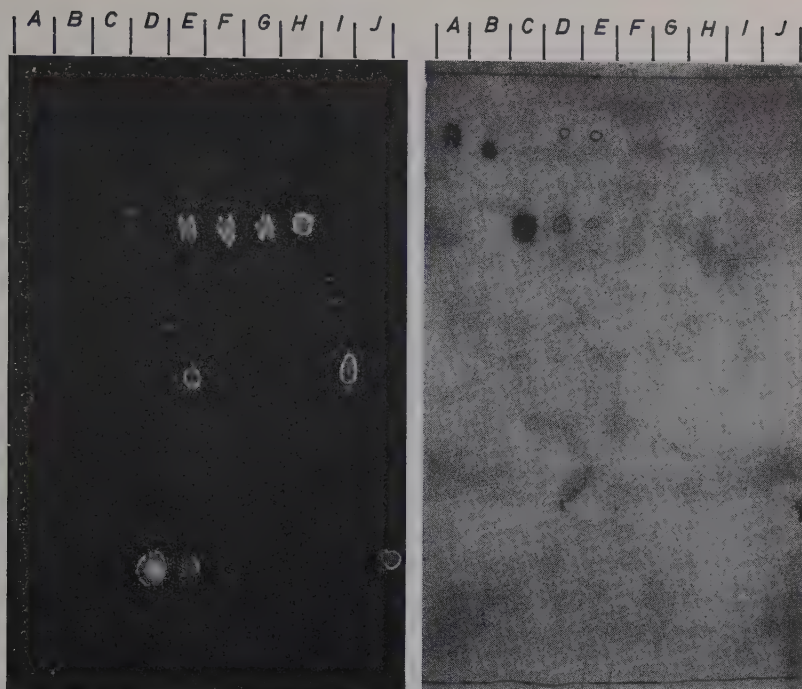


Figure 3. *Chromatogram of acid hydrolysates of II α.* Conditions same as for Figure 1. — A, gentiobiose; B, cellobiose; C, glucose; D, II α (HCl 0.10 N); E, II α (HCl 0.01 N); F, II α (HCl 0.001 N); G, II α (water); H, II α; I, F²; J, scopoletin.

hydrolysis. Also, as with F², glucose was identified in the hydrolysate. However, as is shown in Figure 3, by partial hydrolysis a fluorescent intermediate was obtained which travelled with F² on the chromatogram. In addition a second weakly reducing spot was detected after chromatographing the products of hydrolysis with 0.01 N HCl and to a lesser extent with 0.10 N HCl. Its location, close to the markers gentiobioside and cellobioside, and its absence in 1.0 N HCl hydrolysates suggested that this intermediate reducing spot was a di- or polysaccharide which, under conditions of strong hydrolysis, was degraded to glucose. An aliquot of the II α and 0.01 N HCl hydrolysate was, therefore, chromatographed with markers of the disaccharides lactose, melibiose, gentiobiose, cellobiose and maltose, all of which yield glucose on acid hydrolysis. Development with BAW was prolonged in order to separate, as far as possible, these slowly moving sugars. Figure 4 shows that the hydrolysate intermediate traveled more slowly than cellobiose or maltose but separated close to gentiobiose, melibiose and lactose. Complete hydrolysis of II α did not yield galactose which, under these conditions would



Figure 4. Chromatogram of reducing sugar intermediate from 0.01 N HCl hydrolysate of II α photographed after treatment with alkaline silver nitrate reagent. Solvent: BAW — descending. — A lactose; B, melibiose; C, gentiobiose; D, II α (HCl, 0.01 N); E, cellobiose; F, maltose.

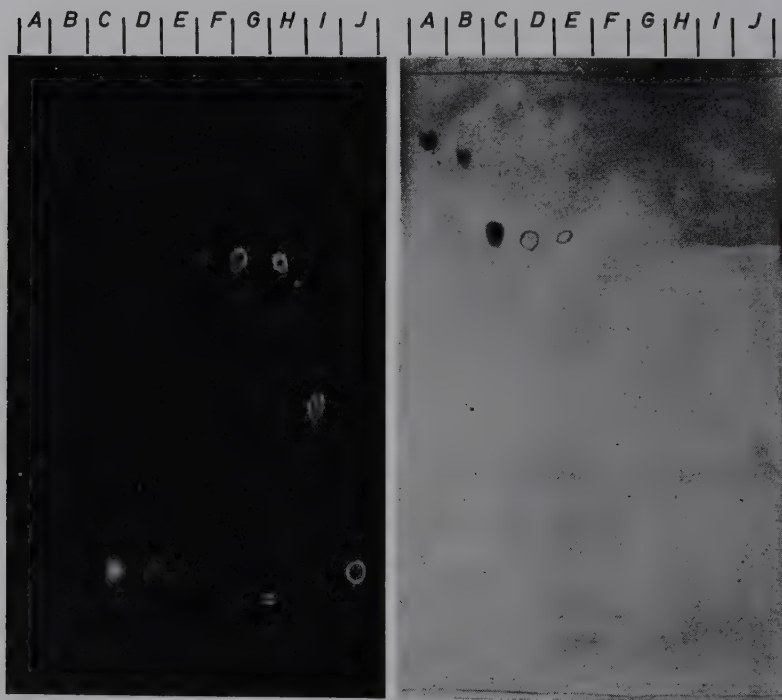


Figure 5. Chromatogram of emulsin hydrolysates of II α . Conditions same as for Figure 1. — A, gentiobiose; B, cellobiose; C, glucose; D, II α (emulsin, 180 min); E, II α (emulsin, 60 min); F, II α (emulsin, 20 min); G, II α (emulsin 30 sec); H, II α ; I, F²; J, scopoletin.

have separated distinctly from glucose. This fact rules out lactose and melibiose as the intermediate.

Emulsin hydrolysis of II α to scopoletin and glucose was readily achieved without the formation of intermediates (Figure 5). On this evidence the α -bonded disaccharides melibiose and maltose must be excluded as possible constituents of the glycoside. Thus it appeared highly probable that II α contains two glucose units β -bonded in series, and that it is, in fact, the β -gentiobioside [6-(β -D-glucosido)-D-glucose] synthesized by Chaudhury *et al.* in 1948, but, until recently found also by Morel (private communication), apparently not reported to occur naturally.

III α

III α , the major constituent of band III, was also hydrolysed with acid and with emulsin and the products analyzed chromatographically as above.

From the results it is apparent that merely heating in water decomposed III α into two components (Figure 6). One was a fluorescent slowly moving

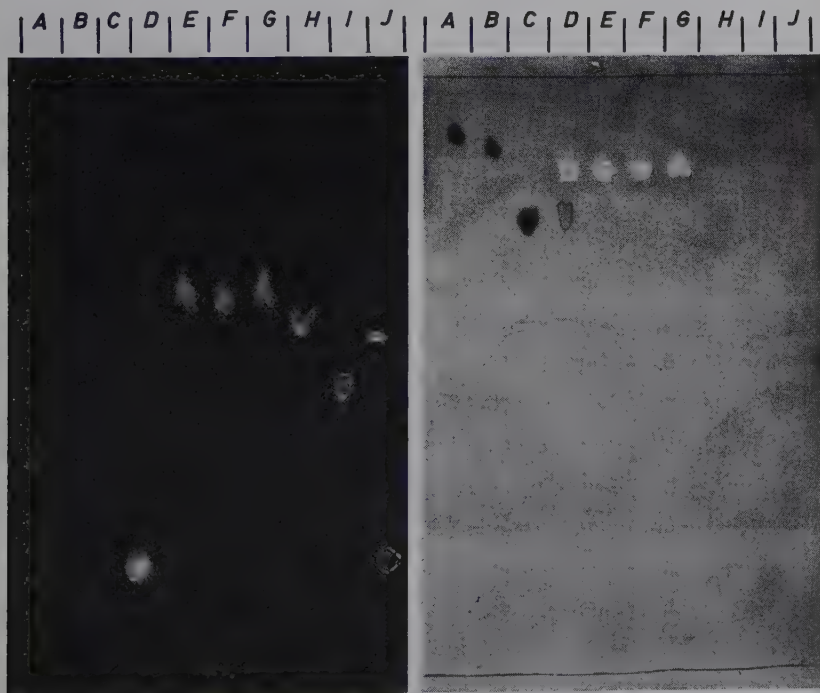


Figure 6. Chromatogram of acid hydrolysates of III α . Conditions same as for Figure 1. — A, gentiobiose; B, cellobiose; C, glucose; D, III α (HCl, 0.1 N); E, III α (HCl, 0.01 N); F, III α (HCl, 0.001 N); G, III α (water); H, III α ; I, F₂; J, scopoletin.

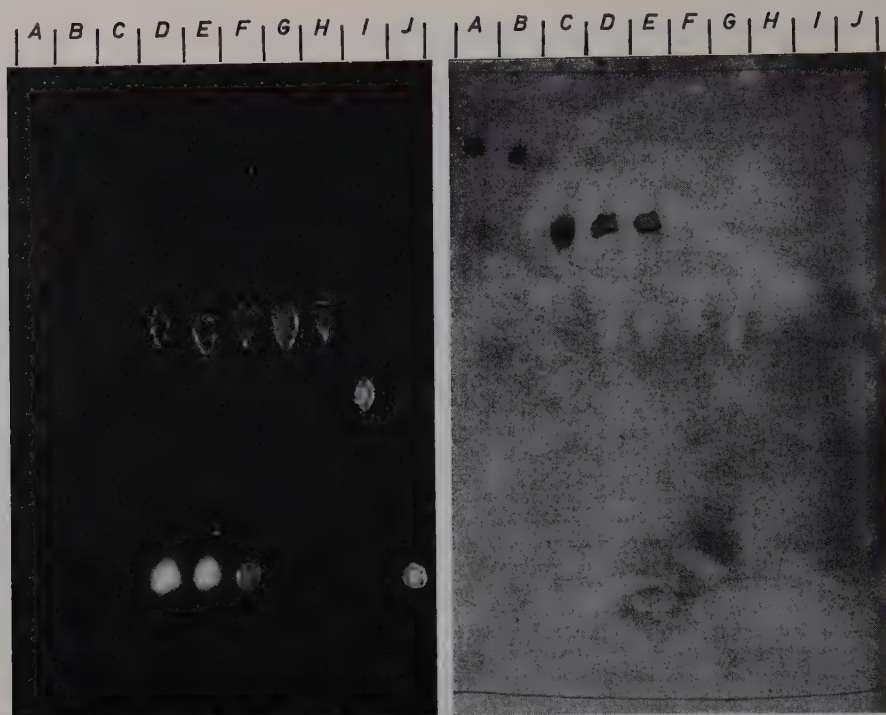


Figure 7. *Chromatogram of emulsin hydrolysates of III α.* Conditions same as for Figure 1. — A, gentiobiose; B, cellobiose; C, glucose; D, III α (emulsin, 180 min); E, III α (emulsin, 60 min); F, III α (emulsin, 20 min); G, III α (emulsin < 30 sec); H, III α; I, F²; J, scopoletin.

substance; the other had no fluorescence, moved slower than glucose, and gave a strong negative reaction with the alkaline silver nitrate, *i.e.*, inhibited the formation of background color. The intermediate fluorescent substance was hydrolyzed to scopoletin and glucose. Very little III α was available for investigating the nature of either of the intermediates but that which reacted negatively towards alkaline silver nitrate was shown to be non-acidic. From this it was concluded that it is strongly oxidizing.

On enzymatic hydrolysis, (Figure 7) III α passed directly to scopoletin and glucose without evidence of the formation of any other substances in terms of fluorescence or positive or negative reaction with alkaline silver nitrate.

Q

The tobacco plants from which fractions F², II α and III α were obtained were harvested in October. In an attempt to work up further quantities of

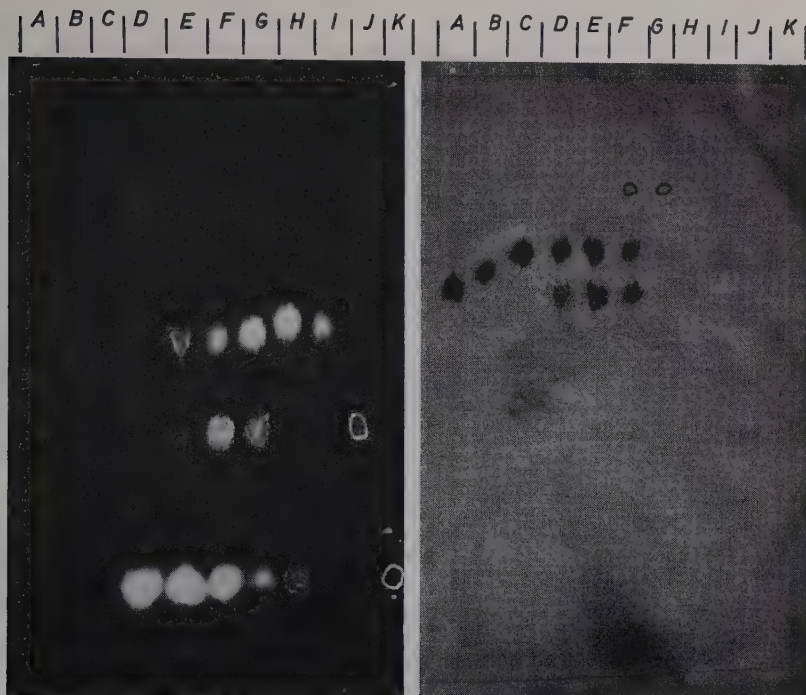


Figure 8. *Chromatogram of acid hydrolysates of Q.* Conditions same as for Figure 1. — A, xylose; B, fructose; C, glucose; D, Q (HCl, 1.0 N); E, Q (HCl, 0.10 N); F, Q (HCl, 0.01 N); G, Q (HCl, 0.001 N); H, Q (water); I, Q; J, F²; K, scopoletin.

II α and III α , more plants were treated in the same way during March of the following year. On chromatographing the ethanolic extract, a strong blue fluorescing band between II α and III α was immediately apparent. Although this band was no doubt present in the earlier extracts, its fluorescence then was weak, and this fraction was rejected during the purification procedure. This band Q, from chromatograms of the second extract was purified and investigated in terms of its hydrolysis products in the same manner as described for the other bands. The chromatograms are shown in Figures 8 and 9.

On acid hydrolysis Q passed to scopoletin via a fluorescent intermediate, probably scopolin. At the same time a number of reducing substances were formed. One of these travelled with glucose and another with xylose. On very mild hydrolysis a third reducing spot appeared which was closer to the origin than glucose. The observations are consistent with the identification of Q as fabiatriin, the β -primeveroside [6-(β -D-xylosido)-D-glucose] of scopoletin, (Chaudhury *et al.* 1948). Hydrolysis of this glycoside would yield glucose and

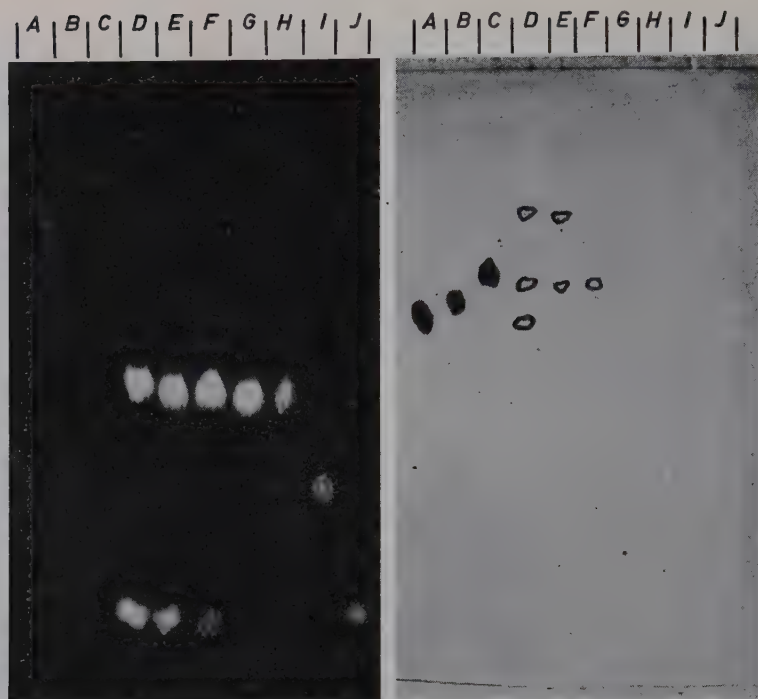


Figure 9. *Chromatogram of emulsin hydrolysates of Q.* Conditions same as for Figure 1. — A, xylose; B, fructose; C, glucose; D, Q (emulsin, 9 hr); E, Q (emulsin, 3 hr); F, Q (emulsin, 1 hr); G, Q (emulsin < 30 sec); H, Q; I, F²; J, scopoletin.

xylose. The latter, on heating with strong acid, passes to furfural and in Figure 8 it can be seen that the xylose spot from Q (HCl, 1.0 N) was weaker than that from Q (HCl, 0.10 N). The weak reducing spot obtained under mild hydrolysis was probably primeverose. A sample of this sugar was not available as a marker but slow movement in this solvent is typical of a disaccharide.

Figure 9 shows the difficulty with which Q was hydrolyzed by emulsin. Even after 9 hr much of the glycoside remained unchanged. It is not surprising, therefore, that the easily hydrolysed scopolin (cf. Figure 3) did not appear as an intermediate in this experiment. The ready cleavage of the β -linkage between the scopoletin and the glucose moiety is further demonstrated in Figure 9, where it is clear that the most abundant sugar in the emulsin hydrolysate was the slowly running "disaccharide" (presumably primeverose). It was, therefore, tentatively concluded that Q is fabiatriin.

Scopoletin and its four glycosides so far isolated have been chromatographed in a number of solvents and the R_f values for these are presented in

Table 1. R_f values of *scoipoletin*, *scopolin* (F^2), *fabiatriin* (Q), *scoipoletin gentiobioside* ($II\alpha$), and *unknown*, ($III\alpha$) in various solvents. Paper: Whatman No. 1. Direction: descending. Origin-Solvent front distance: ca. 35 cm. Temperature: 28°C. Solvents were freshly prepared prior to equilibration.

Solvent	Scoipoletin	Scopolin	Fabiatriin	Scoipoletin Gentiobioside	$III\alpha$
Water	0.50	0.67	0.73	0.75	0.68
CO ₂ -saturated water	0.26	0.64	0.71	0.73	0.67
Acetic acid, 10 %	0.48	0.75	0.78	0.80	0.77
Acetone : Water 9 : 1	0.89	0.60	0.32	0.23	0.39
Ethanol, 50 %	0.77	0.77	0.75	0.75	0.77
Butanol : Water ¹ 1 : 1	0.84	0.45	0.21	0.14	0.24
n-butanol : Acetic acid : Water ¹ 4 : 1 : 2	0.84	0.61	0.47	0.41	0.50
n-butanol : NH ₄ OH ² : Water ¹ 4 : 1 : 5 ..	0.45	0.51	0.26	0.16	0.29
Ethyl acetate : Water ¹ 1 : 1	0.92	0.08	0.00	0.00	0.00
Ethyl acetate : HCl, 2N ¹ 1 : 1	0.87	0.39	0.36	0.27	0.43
Ethyl acetate : NH ₄ OH, 2N ¹ 1 : 1	0.07	0.07	0.00	0.00	0.00
1-pentanol : Acetic acid : Water ¹ 4 : 1 : 5 ..	0.84	0.33	0.11	0.07	0.13
1-propanol : NH ₄ OH ² : Water ¹ 10 : 1 : 1 ..	0.41	0.51	0.28	0.21	0.34

¹ Organic-rich phase used.

² NH₄OH, 28 %.

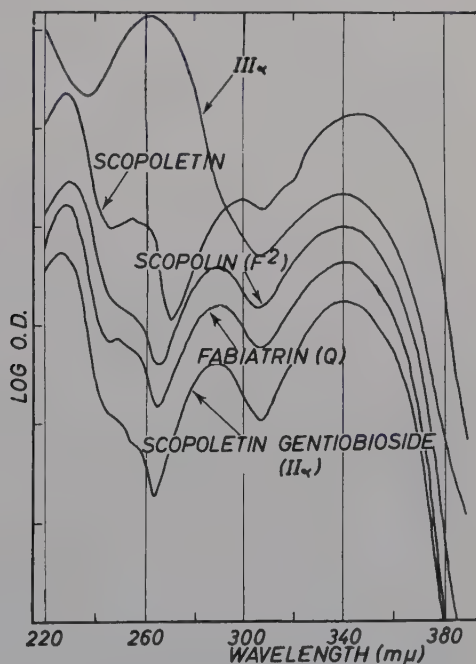


Figure 10. U.v. absorption spectra of *scoipoletin*, *scopolin* (F^2), *fabiatriin* (Q), *scoipoletin gentiobioside* ($II\alpha$), and $III\alpha$. Solvent: 80 % ethanol.

Table 1. In all cases the substances ran as a single fluorescent spot. Each value is the mean of at least three separate chromatographs between which little variation was observed. Whatman No. 1 paper was used and the de-

Table 2. *Maxima and minima in u.v. absorption spectra (220—390 mμ) for scopoletin, scopolin (F²), fabiatriin (Q), scopoletin gentiobioside (II α), and III α. Solvent: 80 % Ethanol.*

Level	Scopo- poletin	Scopolin	Fabiatriin	Scopoletin Gentiobioside	IIIa
Max.	230 mμ	228 mμ	228 mμ	226 mμ	} min. 237 mμ
Min.	246	—	246	—	
Max.	255	—	249	—	} max. 262
Min.	271	265	265	264	
Max.	299	291	290	290	} 307
Min.	307	306	306	307	
Max.	346	340	340	340	340

scending solvent flow was 90° to the direction of milling. The solvent front in each case was allowed to run ca. 35 cm. from the origin.

The absorption spectra (between 220 and 390 mμ) of the five substances in 80 % ethanol are shown in Figure 10. Optical densities are plotted logarithmically, thus allowing the shape of the spectral curves to be compared directly regardless of the concentration of the solutions. With the exception of III α, there is clearly little difference between the position of the maxima and minima of these curves. Even the shape of the III α curve parallels that of the others above 300 mμ. The positions of the maxima and minima are shown in Table 2.

Discussion

Earlier work (Skoog and Montaldi 1961) included an investigation of a scopoletin glycoside obtained from tobacco tissue and known to be identical with that identified as scopolin in the present study. Chromatographic analysis of an hydrolysate of the glycoside during the earlier investigations indicated the presence of two reducing sugars which ran with glucose and fructose respectively. This was consistent with the results of Goodwin and Pollock (1954) who likewise examined a glycoside extracted from roots of *Avena*. Observations presented here, however, clearly show the formation of only one reducing sugar, glucose, during hydrolysis of the glycoside. A probable explanation of this discrepancy lies in the methods employed to extract and purify the glycoside. Both Goodwin and Pollock and Skoog and Montaldi fractionated their extracts on alumina columns and obtained a single fluorescent spot on paper chromatograms of the eluates. In the present study it was observed that chlorogenic acid, almost invariably present in tobacco tissue, ran very close to scopolin on paper chromatograms developed in BAW and only after extended development in this solvent did the

two compounds separate distinctly. Separation of an hydrolysate of chlorogenic acid or scopolin contaminated with this compound on a BAW paper chromatogram, in each case produced a reducing spot which ran with glucose and another which ran only just ahead of fructose. Like scopolin, the purified scopoletin extracted here from tobacco gave no reaction with alkaline silver nitrate. However, both Best (1944) and Swain (1952), who examined a sample of Best's product, reported the reduction of this reagent by scopoletin; as did Burton (1956) who extracted it from potato tubers. Another equally possible explanation of the earlier workers' results may have been contamination of the scopolin with fabiatriin. The latter on hydrolysis yields xylose (see Figure 9) which runs only slightly ahead of fructose in the BAW solvent system. Eberhardt (1955) also examined the fluorescent substances present in *Avena* roots and concluded that the glycoside of scopoletin contained only glucose as the sugar moiety. He hydrolyzed the glycoside with both HCl and β -glucosidase. From his chromatographic data there is little doubt that the glycoside from oat roots (III) is identical with F². He also identified this in the rhizome of *Scopolia carniolica* and found that another fluorescent compound from this tissue ran on an n-butanol: water chromatogram with an R_f 0.22. This may well have been fabiatriin which runs in this solvent at R_f 0.21. Comparisons of R_f values obtained in the BAW solvent system are not feasible because in the present study a mixture of the components was used which differed from that used by earlier workers but which gave somewhat better separation of monosaccharides.

A "scopoletin system" active in the metabolism of healthy tobacco tissues has been proposed (Sargent and Skoog, Skoog and Montaldi), and scopoletin, mainly in the form of its glucoside, scopolin, was considered active in this system. The likelihood of a functional significance of such a system is enhanced by the present findings that scopoletin exists in tobacco in a number of combined forms. Those examined so far are β -glycosides ranging in complexity from the β -glucoside to the β -gentiobioside and the β -primeveroside and one other compound yielding scopoletin, glucose and an unknown oxidising substance. These make up the relatively more abundant fluorescing constituents of the tobacco extract. The existence of still other glycosides is suggested by the minor bands separating on chromatograms during purification of these substances but which have not yet been analyzed.

No detailed studies have yet been made of the levels of these substances within the tissue and their interconversion *in vivo* in response to external conditions. However, seasonal variations in the balance between them seem likely, and changes in the relative intensities of fluorescent bands on chromatograms of crude tobacco extract in response to variations in exogenous auxin/kinetin ratios have been observed.

As to possible physiological functions of these glycosides nothing definite is known. Tryon (1957) working in this laboratory has shown that in clones of tobacco tissue a high scopoletin content was associated with a high capacity for bud formation in that tissue. Various evidence suggests, although as yet none is conclusive, that the family of interrelated scopoletin glycosides in tobacco tissue may function as intermediates in cell wall synthesis, and in this way possibly they influence morphogenesis.

Summary

The more abundant fluorescent substances occurring in ethanolic extracts of tobacco tissue have been examined chromatographically. The presence of free scopoletin has been confirmed and in addition four glycosides of scopoletin have been isolated. Evidence has been presented toward the identification of three of these as scopolin, fabiatriin and the β -gentiobioside. The fourth glycoside, still unidentified, has been shown to yield scopoletin, glucose and an unknown oxidising compound on acid hydrolysis. There are some indications that growth hormone supplies and the seasons influence the relative levels of these compounds within the tissue.

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Growth-promoting and Growth-inhibiting Effects of High Indole-3-Acetic Acid Concentrations

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1. Introduction

It is a well known fact that concentrations of indole-3-acetic acid (IAA) of about 10^{-5} M in 24 hour experiments promote the longitudinal growth of coleoptile sections to a greater extent than do either higher or lower concentrations. Graphically this may be presented as an optimum curve when the longitudinal increment of the coleoptile sections is plotted as a function of the IAA concentration.

In the case of shorter experimental periods the shape of the growth/IAA concentration curve (the concentration curve) is less clear. Thus Foster *et al.* (1952), McRae and Bonner (1953), and Bonner and Foster (1955, 1956) consider it possible to recognize an optimum curve immediately upon the onset of the experiment. They found that the curves representing the growth of the coleoptile sections for different concentrations of IAA as a function of time (time curves) were straight lines. The slopes of these lines increased with increasing concentrations of IAA up to 10^{-5} M after which the slopes decreased with increasing concentrations. Bennet-Clark and Kefford (1954), Hously *et al.* (1954) and Bennet-Clark (1956) do not agree with the above authors in finding the time curves to be straight lines for all auxin concentrations; they find the optimum curve to occur only after a couple of hours growth in the IAA solutions.

The disagreements appear to be due to differences in the experimental

conditions chosen and to different interpretations of the results. Hence only knowledge of the mode of action of the auxin can settle the problem of the shape and location of the time and concentration curves.

The insufficiency of our knowledge in this field does not reflect a lack of attempts to solve the problems. Numerous efforts have been made to elucidate the subject, and among these the studies of Heyn's (1931) are considered of particular importance. They show auxin to have a pronounced effect on the physical properties of the cell wall, in particular enhancing their plasticity. This important discovery has subsequently been confirmed by several authors, among them Ruge, working with hypocotyls of *Helianthus annuus* (1937, 1938), and Burström who was concerned mainly with the extension growth of epidermis cells from wheat roots. Burström reviewed the latter studies in 1957. Both authors base their explanation of the auxin action on a theory assuming the extension growth to be a two phase-process. During the first phase a loosening of the microfibrils in the cell wall takes place simultaneously with a plastic extension of the wall under the influence of turgor pressure. This phase is furthered by addition of IAA. During the second phase an active growth takes place by intussusception of new micelles. According to Ruge the auxin has but little effect on the intussusception processes. Burström who worked with root cells is of the opinion that the reactions during the second phase are probably inhibited by addition of auxin.

The direct, visible result of extension growth is a continuous elongation of the plant part observed. Hence the conception of growth as consisting of two phases with qualitatively different underlying processes appears to be hardly probable unless the two sets of processes proceed more or less simultaneously. This time overlap then must apply also to the reactions connected with the effects of auxin. This causes difficulties when attempts are made to further elucidate the mode of action of auxin on the basis of the two phase-theory. However, it is possible to establish an experimental approximation to the theory of the two phase-growth in the following way. A pre-treatment period (first period) is introduced during which the elongation of the cells is suspended followed by a growth period (second period) during which the increase in length is allowed to proceed uninhibited. By adding or failing to add auxin at will during one or both of the periods it becomes possible to investigate in greater detail the effect of auxin on various growth processes.

A similar procedure has been used previously by Heyn (1931), Thimann (1951), Cleland and Bonner (1956), Cooil and Bonner (1957), and Cleland (1958). Coleoptile sections were used as test material for all of these studies. In no case, however, were experiments made using high concentrations of

IAA, *i.e.*, concentrations which are supra-optimal under conditions giving continuous growth.

In the present paper experiments are described which were designed chiefly to elucidate the effects of such high concentrations on the longitudinal growth of wheat coleoptile sections. The effects of IAA additions were studied under conditions giving continuous growth during the whole experiment as well as by using the bipartite technique described above.

2. Material and Methods

All experiments were made in a thermo-regulated room at 23°C with the exception of those cases where the pre-treatment was made in the cold.

The test material was sections of coleoptiles of the wheat variety Svalöf Progress. Following two hours of soaking in water the wheat grains were sown on wet filter paper in Petri dishes (16 × 4 cm) which were placed in the dark. After 48 hours the plants were watered, and after three days the coleoptiles measured two to three cm. By means of a slicer a ten mm section was cut from each coleoptile at a distance of three mm from the tip. The sections were distributed at random in water. A Zeiss measuring microscope 50 (accuracy 0.01 mm.) was used to measure the lengths. Before transferring the sections to the test solutions the average length was determined for 20 sections. This average length is described as the initial length of the sections and represents zero on the ordinate axis in the various figures. All handling of the coleoptiles took place in weak red light.

Deionized and glass-distilled water was used in preparing the solutions. McIlvaine's phosphate-citric acid buffer (pH 4.6) diluted 1 : 100 was added to all test solutions. Two ml liquid was used per coleoptile. Besides the various additions of IAA the buffer solution was the sole medium used in the experiments concerning continuous growth (Figure 1).

In the experiments involving the bipartite technique the coleoptile sections were placed under conditions causing a suspension of their longitudinal growth immediately following cutting. Such conditions were established by (a) using solutions of a suitable osmotic pressure, or (b) placing the sections at a low temperature (about 3°C) in previously cooled solutions. The necessary osmotic pressure in case (a) was obtained by adding sucrose or mannitol at a concentration of 0.24 M when the growth was to be suspended for a period of two hours, and 0.30 M when treatment was extended to four hours. Minor, fortuitous deviations (approximately ±0.1 mm.) from the initial length occurred after these treatments, *i.e.* the elongation was not zero at the start of the growth period (zero time). However, these deviations proved to be of no measurable importance for the course of the time and concentration curves.

After the pre-treatment the solutions containing the coleoptile pieces were poured into a strainer in which the sections were washed with 2 × 50 ml. of buffer in order to remove adherent sugar, mannitol, and IAA. The sections were then transferred to the sample solution of the second period. For each of the measurements of length during this period we used 20 sections which were discarded after measuring.

at material was not rotated or agitated during any of the treatments. The illustrations represent experiments repeated several times. The data have been statistically analyzed and the statements concerning significance refer to the 95 per cent probability limit.

3. IAA Addition under Conditions Giving Continuous Growth

Figure 1 A shows a number of time curves for the longitudinal growth of coleoptile sections in buffer solutions to which were added IAA at different concentrations. Several characteristic details in these curves may be pointed out.

Firstly, they indicate that auxin concentrations from 10^{-6} to 10^{-3} M do not show considerable differences in their growth-promoting effect during the first 5 hours. However, closer examined, there appears to be increasing growth with increasing IAA concentrations during the first 3 hours. The relative positions of the curves for different IAA concentrations proved to be reproducible although not all of the mean elongations were significantly different statistically. We have tentatively interpreted this evidence as showing that there is no inhibiting effect of high auxin concentrations on elongation during the first few hours of growth. Subsequently, after about three hours the curves cross and the concentration of 10^{-5} M IAA which is normally the optimum one in 24 hour experiments now show a stronger growth-promoting effect than do the others. Similar courses of time curves have been reported for *Avena* coleoptile sections by Cleland (1960) and by Marinos (1957).

Figure 1 A further states that the first part of the time curve for the sections given no IAA treatment curves downwards, while the curves for IAA treated ones show a slight tendency to assume a sigmoid shape. This tendency decreases with increasing concentrations of auxin. These curve shapes are in agreement with the ones found by Bennet-Clark and Kefford (1954) and by Housley *et al.* (1954), but they are in contrast with the linear time curves found for all concentrations by Foster *et al.* (1952), McRae and Bonner (1953), and by Bonner and Foster (1955, 1956).

The time curves in Figure 1 A can proceed in different ways to give the general picture of an optimum concentration curve after 24 hours growth as shown in Figure 1 B. Bennet-Clark and Kefford (1954), Housley *et al.* (1954), and Marinos (1957) have shown with *Avena* coleoptile sections that the time curves for the high concentrations not only proceed with a less positive slope after some hours of growth, but even with a negative slope,

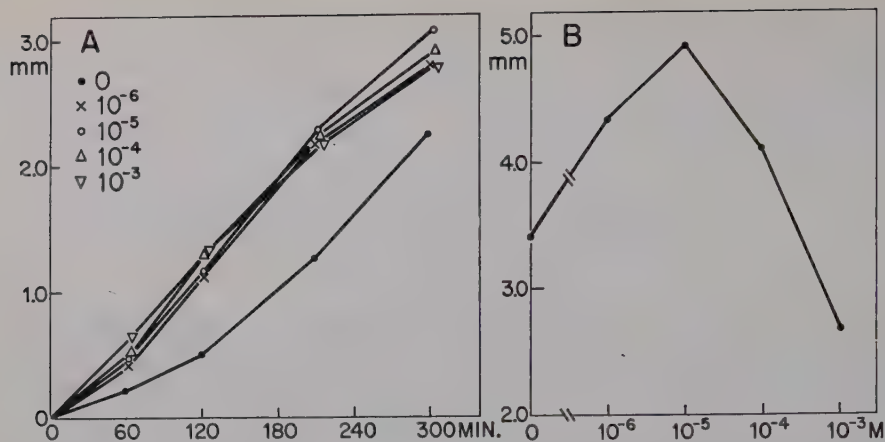


Figure 1. A. Time curves for continuous growth of wheat coleoptile sections at 25°C in citric acid-phosphate buffer with the addition of IAA at the molar concentrations given on the curves. Ordinate: elongation in mm. B. Concentration curve after 24 hours growth in the buffer. Ordinate: elongation in mm. Abscissa: molar concentration of IAA.

i.e. the sections shrink. According to our experience this drastic effect is presumably more pronounced for *Avena* sections than for wheat coleoptile sections.

4. Experiments Including a Period During Which the Elongation of the Coleoptile Sections is Suspended

4 a. Addition of IAA during the first period

The bipartite technique described in section 2 was used in an attempt to study in further detail the effect of IAA on the longitudinal growth. The coleoptile sections were placed for two hours in 0.24 M solutions of sucrose to which had been added IAA in concentrations of from zero to 10^{-3} M. Following this the sections were transferred to buffer solutions and their elongation was measured at intervals for the first four hours. For practical reasons it was impossible to include more than three concentrations of auxin per experiment.

The results obtained are depicted as time curves in Figures 2 A and B. The curves for the low concentrations (2 A) separate at a very early stage. After 20 minutes the differences between them are already significant. For the higher concentrations (2 B) the differences become significant only after a period of 2 hours. However, the most conspicuous fact about these curves

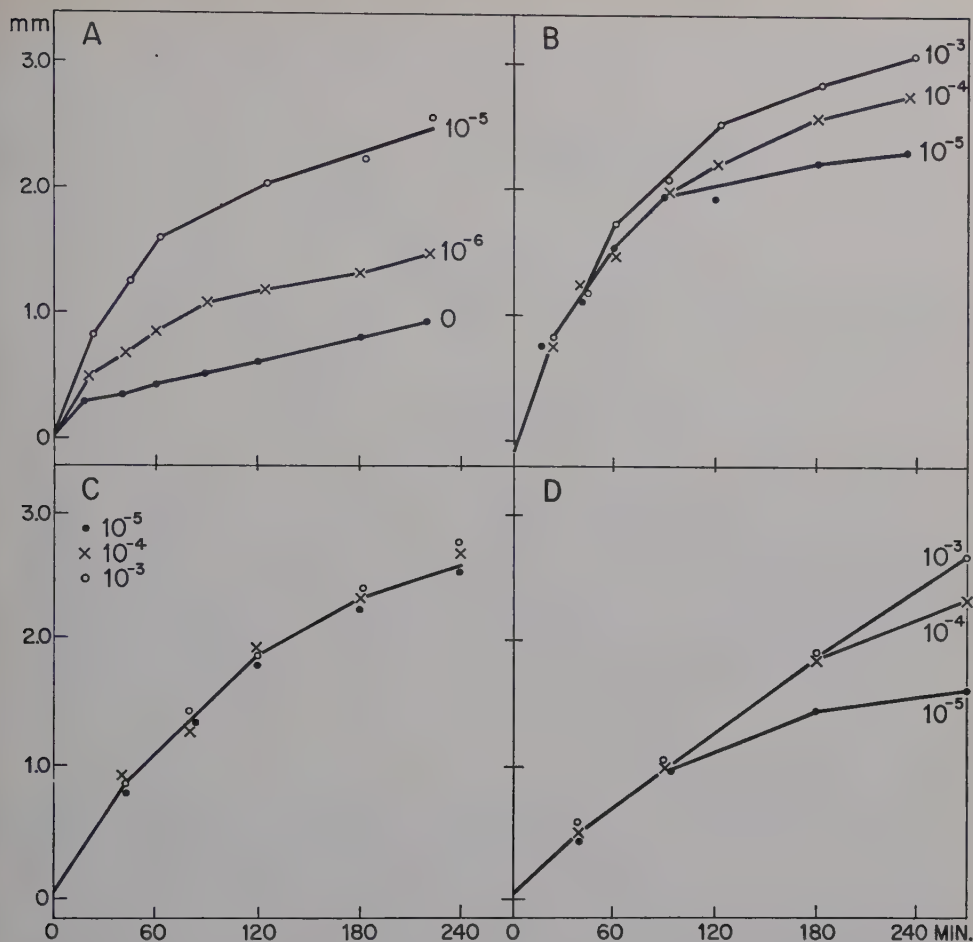


Figure 2. Growth of wheat coleoptile sections in pure citric acid-phosphate buffer at 23°C after pre-treatment involving suspension of growth. Duration of pre-treatment two hours. Growth suspended in A and B by means of a 0.24 M sucrose solution and in C and D by means of a 0.24 M solution of mannitol and by low temperature (3°C), respectively. The numbers by the time curves indicate the molar concentration of IAA during pre-treatment. Ordinate: elongation in mm.

is their sequence. Without exception increasing concentrations of IAA have raised the level of the curves. This means that increasing concentrations have induced increased growth. This was perhaps to be expected up to concentrations of 10^{-5} M. But even 10^{-4} and 10^{-3} M which have proved to be supra-optimum concentrations in other current experimental methods had a higher growth-promoting affect in this case than did the lower IAA concentrations.

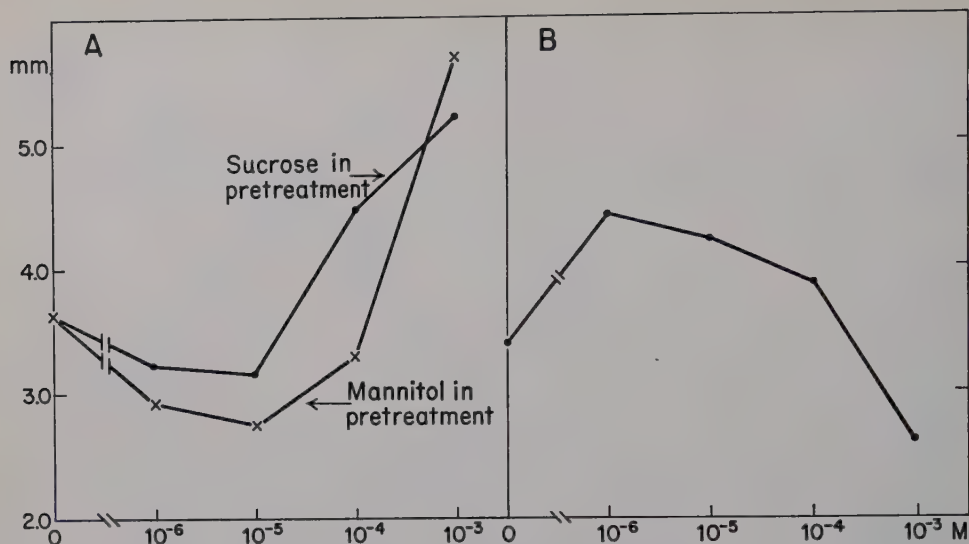


Figure 3. Concentration curve for the growth of wheat coleoptile sections in citric acid-phosphate buffer at 23°C for 20 hours following pre-treatment involving two hours of suspension of growth. Growth suspended in B by means of a 0.24 M solution of sucrose. IAA was added during pre-treatment (A) and during growth (B). Ordinate: elongation in mm. Abscissa: molar concentration of IAA.

The concentration curve after 20 hours of growth during the second period is shown in Figure 3 A. In this case the sequence for the three highest concentrations (10^{-3} , 10^{-4} , and 10^{-5} M IAA) is the same as after four hours. However, for the three lowest concentrations the reverse is the case; this means that the time curves for these concentrations have crossed in the meantime. After treatment with 10^{-5} M IAA the growth is significantly different from the elongation of the control and of the sections treated with 10^{-4} and 10^{-3} M IAA. The reason for the particular shape of the curve was not studied in any further detail.

The time and concentration curves described so far were all established on the basis of experiments in which sucrose was employed as an osmoticum during the first period. In order to determine whether or not the placing of the curves was caused specifically by the sucrose treatment, an identical series of experiments was carried out using mannitol instead of sucrose. This resulted in an absence of significant differences between the time curves during the first four hours of the growth period after pretreatment with 10^{-5} , 10^{-4} , and 10^{-3} M IAA (Figure 2 C). After addition of IAA at concentrations of zero, 10^{-6} , and 10^{-5} M the course of the time curves was similar

to that shown in Figure 2 A. The concentration curve after 20 hours growth (Figure 3 A) was essentially of the same shape as the corresponding one after sucrose treatment. Hence there is no qualitative difference between the effects of sucrose and mannitol treatments on the increase in length. However, sucrose does appear to promote the separation of the time curves for the high IAA concentrations.

In the experiment shown in Figure 2 D the elongation is inhibited by means of low temperature (3°C) during a first period of two hours. The previously found sequence of the time curves during the second period for the normally supra-optimum concentrations of IAA applies here as well. The separation of the curves takes place within four hours.

The first period during which the elongation of the coleoptile sections was suspended in various ways lasted a total of two hours in the experiments described so far. Under conditions giving continuous growth it has previously (Figure 1 A) been shown that two hours from the beginning of the experiment is a time when the time curves for the higher concentrations have a tendency to be the upper ones. In order to decide whether the results found for growth in second period after pre-treatment for two hours with high concentrations of IAA represented an increase in this tendency the elongations during the second period were recorded after pre-treatment periods of 4 and 17 hours using IAA concentrations of 10^{-5} , 10^{-4} , and 10^{-3} M. Sucrose solutions were used as a means of suspending growth for a period of four hours. The course of the time curves during the first four hours of the period of growth was similar to that shown in Figure 2 B, and after 20 hours their relative positions were unchanged.

An attempt was also made to use sucrose solutions as a means of suspending the elongation for a period of 17 hours. However, the ability of the coleoptile sections to grow in the buffer solution appeared to have been considerably reduced by this treatment. Consequently they were placed at a temperature of approximately 3°C during the pre-treatment period instead. However, some elongation resulted during the 17 hours. After transfer to the buffer solution at 23°C the growth was measured after 2, 4, and 22 hours. As may be seen from Table 1 there was a general effect of the addition of IAA, but there appeared to be no difference in the effect of the different concentrations. Placing the sections at 0°C for 17 hours did not appear to suspend elongation for this duration either. In some of the experiments at low temperature growth inhibition set in after the addition of 10^{-3} M IAA.

Hence the results from the experiments with a 17 hour pre-treatment are apparently not in accordance with the results obtained from the other experiments with the bipartite method. Whether this is due to the elongation

Table 1. Growth of wheat coleoptile sections in pure buffer solution at 23°C (second period) after pre-treatment for 17 hours (first period) with different IAA concentrations.

An attempt was made to suspend growth during the pre-treatment by placing the sections at 3°C.

IAA conc. 1st period	Elongation in mm during 2nd period after hrs.			
	0	2	4	22
0	0.18	0.58	0.87	1.74
10^{-5}	0.37	1.08	2.01	2.70
10^{-4}	0.30	1.17	2.00	2.81
10^{-3}	0.32	1.19	1.93	2.81

during the 17 hour pre-treatment or due to the length of this period was not tested.

Burström (1954) made a somewhat similar experiment as ours with only partial suspension of elongation during a long pre-treatment period. For a period of 24 hours 0.3 *M* mannitol was added to the nutrient solution for growing wheat plants. After the 24 hour treatment the length of the epidermis cells of the roots was less when 1-naphtyl-acetic acid (10^{-7} *M*) had been added than when no addition was made. After a subsequent water-saturation, the cells from the auxin-treated roots were still shorter than the corresponding cells from untreated roots. However, the increase in length after water-saturation was the same for the two treatments when computed on the basis of the results given. Hence, there appears to have been no growth-promoting effect of a normally growth-inhibiting auxin concentration in roots either following a long pre-treatment period of only partial inhibition of the cell elongation.

4 b. Addition of IAA during the second period

The elongation of the coleoptile sections was suspended for a two hour first period by means of a sucrose solution. The sections were then transferred to a buffer solution to which had been added IAA. This treatment resulted in the growth shown in Figure 3 B after 20 hours during the second period. The concentration curve has an optimum at 10^{-6} *M* IAA. The peak of the curve is not very pronounced, and some of the replications of the experiment did indeed show an optimum of growth at 10^{-5} *M* IAA. This may be due to a shift in the optimum auxin concentration which is of a nature similar to that shown by Schneider (1938) for continuous growth of coleoptile sections of *Avena* in sucrose solutions to which auxin was added.

Hence after addition of auxin during the second period the high concentrations induced inhibition of growth. A comparison of this with the results

of the experiments concerning dosage of IAA during the first period exclusively (Figure 3 A) shows auxin treatment during the first period to give a curve which is qualitatively different from that resulting from auxin treatment during the second period. In the former case a minimum curve type results, whereas in the later case an optimum one is obtained. This difference in curve types invites the conclusion that there is an effect in first period of IAA added during this period; since, if this were not the case, the resulting concentration curve after 20 hours of growth during the second period would be an optimum curve or at least part of one.

Heyn showed as early as 1931 that provided endogenous auxin during the first period causes increased longitudinal growth during the second one, an effect does indeed exist of auxin already present during the period in which growth is suspended. This has subsequently been confirmed by Cleland and Bonner (1956), *inter alia* using anaerobic conditions during elongation. The limitations of this method were demonstrated by Ordin, Applewhite, and Bonner (1956).

4 c. Addition of IAA during the first as well as during the second period

The elongation of the sections was suspended by means of a sucrose solution during a 2 hour pre-treatment period.

The effect of addition of IAA during both the first and the second period on growth during the second period is seen in Table 2. Concentrations of 10^{-5} and 10^{-3} M IAA during pretreatment are here combined with zero, 10^{-5} and 10^{-3} M IAA during the period of growth. If the elongation of sections treated with IAA in the second period is compared to the elongation of samples not treated with auxin during this period, the following appears from the table: A concentration of 10^{-5} M IAA during the first as well as the second period ($10^{-5} \rightarrow 10^{-5}$) has a growth-promoting effect. $10^{-3} \rightarrow 10^{-5}$ on the other hand induces inhibition of growth. $10^{-5} \rightarrow 10^{-3}$ and $10^{-3} \rightarrow 10^{-3}$ both induce inhibition, the effect, however, being considerably stronger in the latter case than in the former.

Table 2. Elongation of wheat coleoptile sections after 20 hours at 23°C in different concentrations of IAA (second period) following pre-treatment involving suspension of growth (first period). Concentrations of 10^{-5} and 10^{-3} M of IAA were used for pre-treatment.

Growth was suspended by means of a 0.24 M solution of sucrose.

1st period	10^{-5}	10^{-5}	10^{-5}	10^{-3}	10^{-3}	10^{-3}
2nd period	0	10^{-5}	10^{-3}	0	10^{-5}	10^{-3}
Elongation mm (20 hrs.)...	3.25	4.48	2.56	5.23	3.82	2.37

These experiments clearly show that the effect of IAA added during the period of growth depends on the concentration of IAA used during pre-treatment. Hence, there is a certain interaction between the IAA added during the first period and that added during the second one. This appears to support the claim of Cleland and Bonner (1956) that a residual concentration of IAA remains during the second period after pre-treatment with this substance. In any case a residual effect remains.

5. Discussion

The Ruge-Burström theory of the two phases in the longitudinal growth is adopted here because certain of the results obtained are better understood at present by using the explanation offered by this theory on the effects of auxin on the growth during the two phases.

This is true of the fact that no growth-inhibiting effect exists of the supra-optimum concentrations of IAA during the first 3 hours under conditions allowing continuous growth (Figure 1 A). If the increase in length during this period is considered to be the result of the growth processes during the first phase, one would expect to find increasing growth with increasing concentrations of auxin. A tendency towards this effect does appear to exist; the failure of the effect to establish itself significantly may be due to the inexpediency of the experimental conditions.

In order to improve the conditions we introduced the bipartite method. We attempt to correlate its two periods and the two phases of the continuous longitudinal growth in the following way. The loosening of the cell wall which is promoted by IAA during the first phase is considered to take place also during the first period of treatment when increase in length is suspended. This comparison is supported by the fact that Preston and Hepton (1960) found an increased extensibility in coleoptile sections of *Avena* after treatment with both high and low concentrations of IAA while growth was suspended. This indicates that the main difference between the theoretical first phase and the treatment first period consists in the passive plastic extension which is of no decisive importance here. During the second period a plastic extension probably takes place in addition to the formation of new fibrils by incorporation of new material which is characteristic of the theoretical second phase.

With the exception of the experiments with a 17 hour pre-treatment period, the results of which are somewhat dubious, the bipartite method showed a growth-promoting effect during the second period of high IAA concentrations added during the inhibition of growth. Since also an effect of the auxin

was demonstrated during the first period the object in using this method would have been reached had it not been for the fact that an effect was found during the second period as well. However, this was clearly demonstrated by means of the interaction taking place after the addition of IAA during both the first and the second period (Table 2). Hence there is at present no basis for the conclusion that supra-optimum concentrations of IAA promote the growth processes during the first period (alias first phase).

We can hardly conceive of the reverse, *i.e.* an inhibition, taking place during this period. Sooner or later it would inevitable show up in the shape or the position of the time curves, but in fact it occurs neither during their initial course (Figures 2 B, C and D) nor in the general picture after 20 hours growth in the second period (Figure 3 A).

It then remains to maintain that in any case there is no inhibiting effect of the supra-optimum concentrations of IAA up to 10^{-3} M, neither during the first period of the bipartite method (when this is not too long) nor during the first 3 hours under conditions of continuous growth.

Summary

1. In wheat coleoptile sections a tendency was demonstrated towards increasing growth with increasing concentrations of indole-3-acetic acid (IAA) up to 10^{-3} M during the first 3 hours under conditions allowing continuous growth. The harmful effect of concentrations above approximately 10^{-5} M shows up only later during the growth period.

2. By using a bipartite method comprising one period during which the increase in length is suspended and a second period during which elongation takes place, it was possible significantly to demonstrate increasing growth with increasing concentrations of IAA from 10^{-5} to 10^{-3} M after addition of the auxin during the first period. Further, an effect of IAA during this period was demonstrated, and also a residual effect during the subsequent period of growth.

3. It is concluded that there is certainly no inhibiting effect of the supra-optimum concentrations of IAA, neither during the first period of the bipartite method nor during the first 3 hours under conditions allowing continuous growth.

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Studies on the Constitutive Synthesis of β -Galactosidase in a Strain of *Escherichia coli*

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Ordinary wild type strains of *Escherichia coli* will synthesize the enzyme β -galactosidase at a high rate only in the presence of lactose or when supplied with certain other galactosidase which induce the formation of the enzyme. In the absence of such inducers, the rate of β -galactosidase synthesis has been estimated to be only 0.1 per cent of that obtained in cells grown in the presence of the inducer methyl β -thiogalactoside. Soon after the isolation by Cohen-Bazire and Jolit (1) of mutant strains of the organism which synthesize the enzyme at a very high rate even without exogenously supplied inducers, it was observed by Monod and Cohen-Bazire (8) that such a constitutive formation of the enzyme was strongly inhibited by the addition of lactose.

The present investigation was initiated in order to study the effect of lactose on constitutive β -galactosidase synthesis as compared with the effect of certain other compounds. As part of this work, it was discovered that fluoro β -D-galactoside is a substrate of the enzyme, but that it had only a relatively slight effect on the rate of constitutive enzyme synthesis (5). The marked inhibition of growth that was observed when substrates of the enzyme are added to cultures synthesizing β -galactosidase at a high rate was also studied in some detail (6). As a result of these studies and the data presented in this paper, it is assumed that the lactose-effect on constitutive β -galactosidase synthesis is due to a feedback mechanism in which products of the enzyme reaction are further metabolized to a repressor compound, which influences the rate of enzyme formation. Other less specific effects on the enzyme-

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forming system are, however, not excluded, and almost any change in the metabolism of the cells seems to interfere at least temporarily with the rate of β -galactosidase synthesis in the cells.

Various models have been proposed to explain the difference between inducible and constitutive enzyme synthesis in bacteria. Halvorson (4) has recently reviewed the current hypotheses in this field, and only a few questions relevant to the experimental data published in this paper will therefore be discussed in the following.

Material and Methods

The microbiological background of the β -galactosidase synthesis in *E. coli* has been reviewed by Cohn (2). A detailed discussion of the experimental procedures employed in investigations of this kind and relevant references to the properties of the enzyme system are also found in this review.

Strains of Escherichia coli

The β -galactosidase constitutive strain ML 308 was obtained from Dr. M. Cohn. It was maintained lyophilized or on tryptose agar slants. During one stage of this investigation it was found that cultures gave considerably lower differential rates of β -galactosidase synthesis than they had given before under similar conditions of growth. Different cell clones were then isolated from single cell colonies on agar, and one of these which had a similar high level of β -galactosidase activity as the original strain was used for the remaining experiments. Other clones which synthesized the enzyme at a rate of 10–50 per cent of the maximal value obtained with the original strain were also found.

Culture conditions

Cultures were grown in Erlenmeyer flasks incubated on a rotatory shaking machine in a water bath maintained at 37°. The inorganic-salts medium "56" (Monod *et al.* 9) was used, and carbon sources were added as sterile solutions or as solids in the case of short term experiments. The thiogalactosides were gifts from Dr. M. Cohn; other compounds were of commercial origin. Exponentially growing cultures were obtained by inoculating a medium containing a large excess of a carbon source (usually 0.4 per cent) with an overnight culture grown in a similar medium containing only 0.1 per cent of the carbon source. After two or three divisions of exponential growth, the culture was again diluted into a similar medium at the start of the actual growth experiment.

Determination of bacterial growth

The absorbancy of withdrawn samples of growing cultures was determined in an Eppendorf electrophotometer using 1 cm. semimicro cuvettes and a 405 m μ inter-

ference filter. Only cultures of low cell densities (less than 5×10^8 cells per ml) were used so that direct proportionality between the absorbancy reading and bacterial cell mass was obtained. An absorbancy of 0.1 was found to be equivalent to approximately 30 μg dry weight or 1.2×10^8 cells pr ml during exponential growth.

Enzyme activity determinations

The technique described by Monod *et al.* (9) was used. However, toluene treatment of the cells was found to give reproducible values only when exponentially growing cells were assayed. Novick and Weiner (12) found that the addition of both toluene and 10 μg . deoxycholate per ml. cell sample gave more uniform results. This method was therefore adopted, and it was found to give up to thirty per cent higher enzyme activity values than toluene treatment alone, when lactose-inhibited cells were assayed.

One unit of β -galactasidase activity is that amount of enzyme which hydrolyses 1 μmole of *o*-nitrophenyl β -D-galactoside in one minute at 28° in the presence of 0.025 *M* substrate and 0.1 *M* sodium phosphate buffer at pH 7.0.

Results

When toluene-treated cells of the strain ML 308 are assayed for β -galactosidase, particularly high activities are obtained in cells growing exponentially in a medium where succinate is the sole source of carbon. Similar high levels of enzyme activity are obtained in cells grown on fumarate, acetate, maltose and glycerol, whereas lactate, glucose, galactose and lactose support the growth of cells with considerably less β -galactosidase activity. In order to obtain comparable data on the specific activity of cells grown on some different sources of carbon, samples of exponentially growing cultures that had been maintained in the respective medium for a large number of cell-generations were assayed (Table 1).

Table 1. *Specific β -galactosidase activity of exponentially growing cells of E. coli ML 308. Values are given as units of enzyme activity per μg . dry weight of bacteria harvested after growth for at least ten generations on the source of carbon indicated.*

Source of carbon for growth in medium "56"	Specific activity
Succinate	15 — 18
Fumarate	16
Acetate	14
Glycerol.....	15
Lactate	13 — 14
Glucose	6 — 7
Galactose	7 — 9
Lactose	5 — 6
Maltose	11

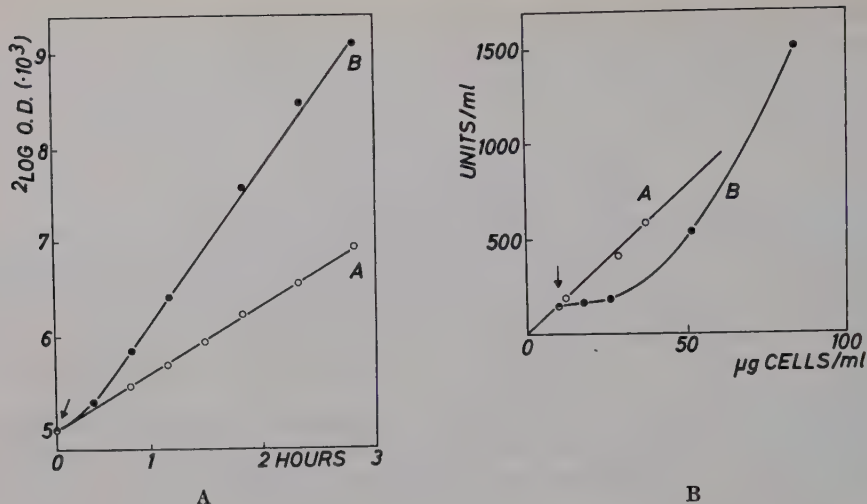


Figure 1. Growth (A) and differential rate of β -galactosidase synthesis (B) of two cultures of ML 308 growing exponentially on

A. Succinate.

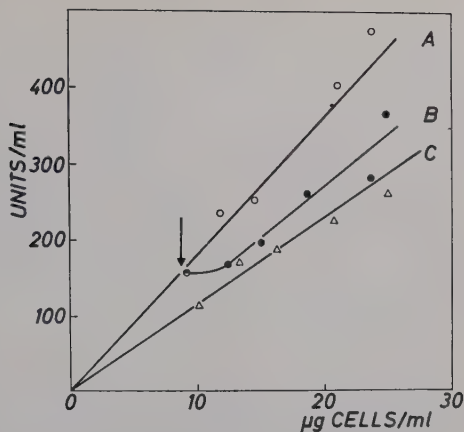
B. Succinate + 1 per cent Difco Bacto Peptone added at the points indicated by arrows.

If the influence of varied culture conditions on the enzyme synthesis is studied, it is convenient to plot the increase in enzyme activity of a growing culture against the increase in cellular material. This will permit an estimation of "the differential rate of enzyme synthesis" (10). This expression is particularly informative when it is desirable to distinguish between the effects of various compounds on the rate of growth and on the rate of enzyme formation. As an illustration, Figure 1 shows the effect of a peptone on a culture of ML 308 growing exponentially in the medium "56" with succinate as the source of carbon. The immediate sharp rise in the rate of growth was not accompanied by a corresponding increase in the rate of synthesis of β -galactosidase. The specific enzyme activity of the cells will under such conditions fall considerably soon after the addition of the peptone. However, the inhibition of the β -galactosidase synthesis was only temporary, and the succeeding rate of enzyme formation was so high that the specific enzyme activity of the stationary cells was even slightly higher than that of cells grown on succinate alone. Similar results were obtained when one per cent vitamin-free casein hydrolysate was added to a culture growing on succinate. Single amino acids such as glutamic or aspartic acid, asparagine and tryptophan had little effect at a concentration of 0.1 per cent.

The addition of glycerol, which supports a constitutive β -galactosidase formation of a similar magnitude as succinate, was also found to cause a

Figure 2. *Synthesis of β -galactosidase on succinate and lactate.*

- A. Succinate only.
 B. Succinate, with 0.4 per cent lactate added at point indicated by arrow.
 C. Lactate only.



marked, but temporary inhibition of the enzyme formation on succinate, and the same phenomenon was observed when maltose was added to a succinate culture. Lactate was found to increase the rate of growth of a succinate culture by approximately fifteen per cent. In this case, the differential rate of β -galactosidase synthesis was decreased to a value similar to that of a culture grown on lactate as the sole source of carbon (Figure 2).

The inhibitory effect of glucose on the synthesis of a large number of enzymes is well-known, and the drastic inhibition of the β -galactosidase induction has been studied in great detail by Cohn and Horibata (3). Monod and Cohen-Bazire (8) reported that a 30—35 per cent inhibition of the differential rate of synthesis was obtained when glucose was added even at relatively high concentrations to a culture of the constitutive mutant ML 308 grown on maltose. When the same strain was grown on succinate, the present author obtained a stronger inhibition, which was dependent on the amount of glucose added as shown in Figure 3.

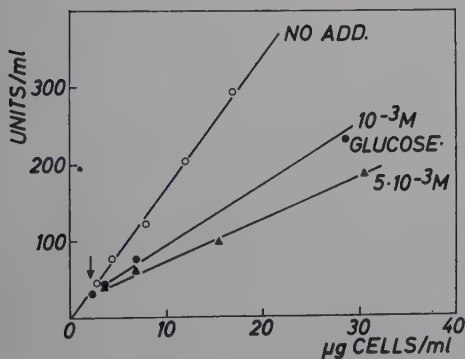


Figure 3. *The effect of glucose addition on the enzyme synthesis by a culture growing on succinate.*

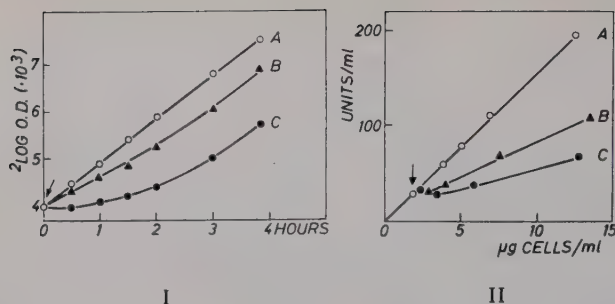


Figure 4. The effect of lactose and galactose on the growth (I) and differential rate of enzyme formation (II) of ML 308 growing on succinate.

A. Succinate only.
B. Succinate + galactose $5 \times 10^{-3} M$
C. Succinate + lactose $5 \times 10^{-3} M$

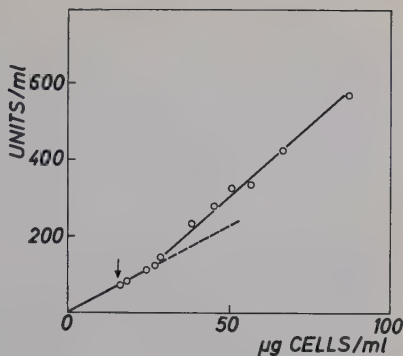
Figure 4 shows that the addition of lactose to a succinate culture of ML 308 brought about a temporary but complete inhibition of the growth, and the succeeding rate of β -galactosidase synthesis was found to be very low. As discussed in more detail by the author in reference 6, the strong growth-inhibitory effect of lactose is dependent both on a very high β -galactosidase activity of the cells and on an effective concentration mechanism (permease) for β -galactosides. An equimolar concentration of thiodigalactoside, which is an inhibitor of the β -galactoside permease was thus found to reverse completely the effect of lactose. Very slight inhibition of the constitutive rate of enzyme synthesis was observed when lactose was added to cells grown on galactose or glucose.

Galactose has a definite inhibitory effect on both the growth and enzyme formation of a succinate culture as shown in Figure 4. As already mentioned, fluoro β -D-galactoside is a substrate of the β -galactosidase, and it is rapidly concentrated into the cells by means of the constitutive permease of the strain ML 308. It is growth-inhibitory on a culture grown on succinate, but it was not found to inhibit the β -galactosidase synthesis more than galactose at equivalent concentrations.

Methyl β -D-thiogalactoside and isopropyl β -D-thiogalactoside, which are very good inducers of β -galactosidase formation in the wild-type strain ML 30, were not found to increase the rate of enzyme formation of the constitutive strain ML 308 if it was grown either on succinate or on carbon sources which gave lower levels of enzyme activity.

It is of interest to note that glucose, galactose and lactose are inhibitory to the β -galactosidase synthesis only as long as they are present in relatively high concentrations. Toward the end of a complete growth cycle in a medium where these compounds are present in growth-limiting amounts, the specific β -galactosidase activity was found to rise to a value which was about three times as high as that obtained during exponential growth on an excess of the carbon source. Similar effects were not obtained with succinate, on which

Figure 5. Synthesis of β -galactosidase during recovery from phosphate starvation. A culture was first grown on excess succinate until all the phosphate available (50 μ moles P_i per ml.) had been exhausted. Such cells were then used to inoculate a medium containing 1 μ mole P_i per ml. at the point indicated.



the specific activity was found to be relatively constant throughout a complete growth cycle.

Some observations on the constitutive β -galactosidase synthesis that were made by the author (7) during studies on the formation of acid and alkaline phosphatase by the strain ML 308 may also be mentioned at this place. The synthesis of alkaline phosphatase is strongly repressed by inorganic phosphate in contrast to that of acid phosphatase, which is present during all conditions of growth. Cells grown in a medium, in which 0.1 *M* Tris-buffer was used instead of the inorganic phosphate of medium "56", and where a low concentration of phosphate had been growth-limiting, were found to have a specific β -galactosidase activity of only fifty per cent of that obtained in the presence of excess phosphate. Figure 5 shows the differential rate of β -galactosidase formation in a culture where such phosphate-starved cells were used to inoculate a medium containing excess phosphate. There was a lag of about one cell division before the cells had reached the higher differential rate of enzyme synthesis that was subsequently characteristic of the culture.

Discussion

The experiments reported show that many apparently unrelated compounds may affect the rate of synthesis of β -galactosidase by a constitutive strain of *E. coli*. For the interpretation of such effects, it is important to be able to distinguish between inhibitory or stimulatory effects on the rate of growth of the cells and of more specific effects on the formation of the enzyme. An estimation of the "differential rate of enzyme synthesis" is the most useful expression when the influence of various additions on a growing culture is studied. In point of language, this expression, which was originally coined by Monod *et al.* (10), is somewhat confusing in that it is equivalent to the ratio between two rates — that of enzyme synthesis and that of cell

growth — but it has become a generally accepted term, and it is often a more informative measure than specific activity data.

On the basis of genetic recombination experiments, Pardee *et al.* (13) have suggested that mutation from wild type inducible cells to β -galactosidase constitutive strains is accompanied by a loss of a specific repressor mechanism (perhaps involving a galactosidic residue). According to the generalized repressor hypothesis (16) the rate of enzyme synthesis is regulated by internally synthesized, specific repressors for which inducers are *antagonists*. This model is relatively simple and attractive and is supported by observations on several enzyme systems (see the review by Halvorson 4). The chemical nature and site of action of the repressors is still a matter of speculation, and the general validity of this mechanism for protein synthesis has also been questioned (15). The existence of strains with different levels of constitutive β -galactosidase activity observed by the present author, and also reported for other enzyme systems by Halvorson (4) and Pollock (14), shows that mutation may lead to only a partial inactivation rather than to a complete absence of the specific repressor mechanism.

The relatively large variations in the β -galactosidase content of the strain ML 308, obtained when it is grown on different sources of carbon, implies either that different metabolites may affect the enzyme-forming system for β -galactosidase, or that these are transformed into one common specific repressor compound. The very high rate of β -galactosidase synthesis obtained on citric acid cycle intermediates would then be due to the absence of appreciable concentrations of such repressor compounds. The rate of growth under these conditions may also be largely limited by lack of carbohydrate intermediates for cell wall synthesis etc., and this would allow a considerable "luxury" production of certain enzymes. Similar differences in the capacity for constitutive β -glucosidase synthesis in yeast has been observed by Halvorson (4), who has also found that these variations are not affected by the addition of inducers or paralleled by differences in the rate of growth.

The observed effect on the differential rate of β -galactosidase formation by the addition of various compounds to succinate-cultures of ML 308 are probably complex in nature. Competition between different enzyme-forming systems for energy and common building blocks may play a role, when a large number of new enzymes are induced by the addition of *e.g.* glycerol or a mixture of amino acids. Inhibition by the formation of a repressor compound is, however, probably operative when carbohydrates are added. Glucose inhibits the formation of most enzymes that catalyze reactions in which glucose or related compounds are formed, and it has been suggested that glucose may be a preferential metabolic source of several internally synthesized repressors (11). The effect of glucose on the β -galactosidase forma-

tion is less drastic in constitutive than in wild type inducible strains. It is nevertheless of such a magnitude that it may be due to the formation of a repressor, but that this accumulates in a lower concentration, or that the enzyme-forming system is less sensitive to its action in the constitutive strains. In yeast, constitutive and inducible β -glucosidase synthesis is equally sensitive to glucose effects (4).

The marked inhibitory effect of lactose on the β -galactosidase formation in the constitutive strain is probably caused by the exceptionally high intracellular concentrations of glucose and galactose obtained when lactose is added. Cells grown on succinate have very high β -galactoside permease and β -galactosidase activities. It is likely that the inhibition of the growth which is obtained when substrates of the enzyme are added to such cells is largely due to the extreme osmotic conditions created in the cells (6). During the recovery from the growth inhibition, the cells may be unable to carry out synthetic reactions at a rate equivalent to the production of catabolic intermediates from galactose and glucose, leading to an accumulation of a repressor operating on the β -galactoside-forming system.

Summary

The constitutive synthesis of β -galactosidase by a mutant strain of *E. coli* was studied under varied nutritional conditions. The advantage of expressing the experimental data in a way that permits comparisons of the differential rate of enzyme synthesis is stressed by the observation that compounds may have widely different effects on the rate of growth and on the rate of β -galactosidase formation. The exceptionally high rate of enzyme synthesis by cells grown on succinate is inhibited by lactate, lactose, glucose and galactose. Other compounds such as maltose, glycerol and amino acids also cause a marked but only temporary inhibition of the β -galactosidase synthesis, when they are added to succinate cultures.

The observed effects on the rate of enzyme formation are discussed in relation to the present knowledge of the mechanism of regulation of enzyme synthesis. The exceptionally strong inhibitory effect of lactose is assumed to be due to a feedback mechanism in which one or several products of the metabolism rather than lactose itself act as repressors of the enzyme formation.

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The Auxin Requirement and the Effect of an Auxin-Antagonist on Tumorous and Normal Tissues of *Picea glauca*

By

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Cultures of a tumor of *Picea glauca* were first made by White and his co-workers several years ago (White and Millington 1954, Reinert and White 1956), and similar cultures, though derived from new isolations, have been carried in this laboratory for several years. It has been found by experience that these tumor tissues in culture are strictly auxin-dependent, whereas cultures from normal tissues, growing much more slowly, do not require auxin at all. This auxin dependence of tumor, but not of normal tissue, is exactly opposite to the results obtained with normal tissues and several types of tumors on a number of plant species (Braun 1947, De Ropp 1947, Jablonski and Skoog 1954, Kulescha and Gautheret 1948, Morel 1948). For this reason, it seemed desirable to establish the auxin requirements as clearly as possible. Reinert and Schraudolf (1959), also using *Picea glauca* tissues, reported that normal tissue grew without auxin, but they found a 50 % promotion at the optimum concentration of IAA.; our own data show no such promotion.

Materials and Methods

The medium for these experiments has been greatly improved and simplified from that of Reinert and White (1956). The tyrosine which caused so much blackening and toxicity in their cultures has been omitted altogether, with greatly improved results. Furthermore, it has been found that, for tumorous tissue at least,

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all amino acids can be omitted without any decrease in the growth rate. Asparagine has been included in the medium though its necessity has not been proved. The composition of the medium, in which the inorganic constituents have also been greatly modified, can be summarized as follows (all concentrations in mM); —

Ca(NO ₃) ₂	0.85	Ferric D-tartrate	0.017	Thiamine	0.00060
KNO ₃	0.50	Inositol	0.55	Riboflavin	0.00053
MgSO ₄	0.41	Asparagine	0.15	Biotin	0.00020
KH ₂ PO ₄	0.37	Choline	0.071	Indoleacetic acid	0.00060
MnSO ₄	0.03	Nicotinic acid	0.0081	Sucrose was added to a	
H ₃ BO ₃	0.024	Ca-pantothenate	0.0042	concentration of 2 % and	
ZnSO ₄	0.0094	Ascorbic acid	0.0023	the pH adjusted to 5.6.	

The methods of culturing and measuring have been described elsewhere by de Torok and Roderick (1961). The choice of auxin and of antagonist are discussed below.

Results

The growth rates of normal and tumorous tissues of *P. glauca* over a 4-month period with and without indoleacetic acid are compared in Table 1. The data are typical of results obtained over a period of several years.

It will be noted that the effective concentration of indoleacetic acid is about 20 times lower than that found necessary by Reinert and White (1956), and Reinert and Schraudolf (1958); this is probably due to the improvement in the medium. The data show that an auxin requirement for cultured tumorous tissues of *Picea glauca* is apparently absolute. The results could, of course, be interpreted as due to a much greater efficiency of auxin uptake by tumorous tissues than by normal, but this is rendered improbable by the fact that at increased auxin levels, the growth rate of the normal tissue was not further increased. Even at as high a concentration as 500 mg per liter of 2,4-D the normal tissues grew only from 250 to 380 mg in 7 months, which is much less than in Table 1. Experiments with other auxins, none of which was any more effective than IAA, will be described in a later paper.

Table 1. *Growth made by tumorous and normal tissues of Picea glauca with and without auxin.* For medium see Materials and Methods. Each figure is the average fresh weight in milligrams of twenty cultures.

Date of measurement	Tumor tissues		Normal tissues	
	IAA 5.9×10^{-7} M	No auxin	IAA 5.9×10^{-7} M	No auxin
Feb. 5, 1960 (initial).....	250	250	250	250
Mar. 5, 1960	3,026	no increment	389	374
Apr. 5, 1960	7,092	dead	432	456
May 5, 1960	11,027	—	621	600
June 5, 1960	16,000	—	800	812

In order to render the auxin effect still more unequivocal, experiments with an auxin antagonist were carried out. For if the tumorous tissue were really dependent on auxin, it would be expected that the application of an auxin antagonist would prevent its growth in presence of optimal auxin. On the other hand, an auxin-independent tissue would not show any marked effect when an auxin antagonist is added to its nutrient.

A number of substances have been described as auxin antagonists (see Åberg 1956), but most of these have some auxin activity in one or another test. However, *p*-chlorophenoxy-*isobutyric* acid (pCiBA) is among those with the smallest growth-promoting activity, and was therefore selected. This substance was added to the medium above in concentration equal to that of the auxin, which was IAA at 5.9×10^{-7} M.

The results are presented in Table 2.

Table 2. *Growth of tumorous and normal tissues of Picea glauca on media containing: 1) IAA, 2) no auxin, 3) IAA plus p-chlorophenoxy-isobutyric acid (pCiBA). Each figure is the average fresh weight in mg of ten cultures.*

Date of measurement	IAA 5.9×10^{-7} M	No auxin	IAA plus pCiBA 5.9×10^{-7} M
Tumor tissues			
Dec. 3, 1960 (initial)	1,000	1,000	1,000
Jan. 3, 1961	3,265	Almost dead	1,200
Feb. 3, 1961	6,427	Dead	1,362
Mar. 3, 1961	8,940	—	1,400 ¹
Normal tissues			
Dec. 3, 1960 (initial)	1,000	1,000	1,000
Jan. 3, 1961	1,347	1,298	1,300
Feb. 3, 1961	1,550	1,543	1,560
Mar. 3, 1961	1,612	1,600	1,592

¹ Average of five, five having been removed for other work.

The growth rates are on the whole very comparable with those shown in Table 1. It will be seen that the tumorous tissues on the auxin media grow from 200 to 300 per cent monthly; in the absence of auxin, no growth occurs and the tissues eventually die, as in Table 1. Correspondingly, the normal tissues grow to almost the same extent (15–20 % per month) with or without auxin.

Column 3 shows the effect of pCiBA. The tumorous tissues show a striking growth inhibition, amounting to some 95 %. The normal tissues, exposed to the same concentrations of IAA and pCiBA, show no inhibition at all; it is evident that there is no antagonism.

It is noteworthy that growth of the tumor culture does continue in presence of the auxin antagonist, and indeed the rate is comparable to that of the normal tissue. The appearance of the culture was not abnormal in any way and the tissues did not die. The incidence of mitoses, checked by the squash method (see de Torok and White 1960) was approximately 12 per 1000 cells as compared to 179 per 1000 cells in the uninhibited tumor tissue. Thus the auxin antagonist has inhibited mitosis by about 93 %, which agrees with the figures above, based on increase in weight. In the uninhibited normal tissue the same method yielded a value of 19 per 1000 cells.

When tissues were removed from the pCiBA to normal IAA medium, the growth rate increased steadily, after some lag. Five tissues averaging 1370 mg. reached in four successive months average weights of 2138, 3070, 5356, and 8138 mg; the last increment was about equal to that of an untreated culture. Recovery is therefore complete. In other experiments using pCiBA at concentrations up to 13^{-3} M, growth of the tumor tissues could be reduced to about 2 % per month; nevertheless the tissues remained alive.

The present case represents one of the very few which are known where a tumorous growth on plants can be inhibited almost indefinitely, without toxicity, by a single chemical. The nearest approach is probably given by the experiments of De Ropp (1951) with a group of folic acid antagonists, which inhibited the growth of *Helianthus* tumor *in vitro*; A-Denopterine at 0.1 mg/l (ca 2×10^{-7} M) was the most effective. The cultures were carried only for 4 weeks, while the inhibitions in Table 2 were obtained over a period of 3 months. The large volume of work on animal tumors (see Ciba Symposium 1958) lies outside the scope of this paper.

Summary

The effect of indoleacetic acid and of an auxin antagonist has been studied on tumorous and normal tissues of *Picea glauca* in culture. The cultures of tumor tissue would not grow in the absence of auxin, and eventually died. Normal tissues, on the other hand, grew equally well with or without added auxin. Furthermore, the auxin antagonist, *p*-chlorophenoxy-*isobutyric* acid, supplied at the same concentration as the IAA, strongly inhibited the growth of tumor tissue, while the growth of the normal tissue was unaffected. Mitoses were also decreased proportionately. These results confirm that the difference between the auxin relations of tumor and normal tissues of *Picea glauca* is the opposite of that for other such pairs of tissue in the literature.

Finally, the simultaneous presence of both auxin and antagonist does permit some limited growth of the tumorous tissue with continued mitoses and without signs of abnormality.

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Effect of Electrical Currents on the Transport of Radiocalcium (Ca^{45}) in *Phaseolus vulgaris*

By

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Introduction

Calcium is one of the most intriguing nutrients in studies of foliar absorption, transport and redistribution of minerals in plants. After initial deposition it remains essentially immobile (Biddulph 1954, Bukovac and Wittwer 1957). Similarly, there is little or no transport of Ca^{45} through graft unions of herbaceous plants involving reversed polarity (Bukovac 1957). Chemical treatments that reportedly reverse polarity also result in some basipetal transport of calcium, but not necessarily through the phloem (Bukovac, Wittwer, and Tukey 1956, Kessler and Moscicki 1958, Biddulph, Cory, and Biddulph 1959). The existence of electromotive forces in plant tissues has been long recognized (Rosene and Lund 1953). Modification of bioelectric potentials by indole-3-acetic acid and the influence of indole-3-acetic acid on ion absorption and transport of radiophosphorus has recently been suggested by Opritov (1958). Results of a study of Ca^{45} movement as influenced by electrical polarity are herein reported.

Methods and Materials

Seeds of *Phaseolus vulgaris* (variety Blue Lake, Stock No. 42335, Rogers Bros., Twin Falls, Idaho) were germinated in coarse No. 8 quartz sand (American Graded Sand Co., Chicago). After unfolding of the primary leaves uniform seedlings were transplanted into standard 4-inch clay pots of loam soil. Within 24 hours after

transplanting, and at the time the primary leaves were only partially expanded, 10 microliters of $\text{Ca}^{45}\text{Cl}_2$ solution (100 microcuries Ca^{45} per milliliter per 3 milligrams of CaCl_2) were applied to the epicotyl tips in such a manner that the solution was dispersed equally in the adjacent axils of each of the primary leaf petioles. Each of eight intact bean seedlings treated in the above manner were then subjected to electrical potentials inducing various rates of direct current flow in the plant tissue. An equal number of Ca^{45} treated plants not subjected to electrical treatment served as controls in each experiment.

The electrophoretic unit consisted of a negative contact made to the tip of one of the primary leaves and a positive contact made to the tip of the opposite primary leaf. Copper electrodes were attached through a paste of finely divided water moistened charcoal ("Norite A", Fisher Scientific Co., Chicago) applied to about one square centimeter of the upper surface of the primary leaf tips. Thus, the influence of an electric current from the tip of one leaf to the other, via the petiole and stem tissue on the transport of Ca^{45} into the two leaves from the application site in the adjacent leaf axils could be determined. Duration of the electrical stimulation in each experiment was seven hours and the current was imposed immediately following the application of radiocalcium. All plants were harvested after 24 hours and partitioned as follows: the blade of the leaf in contact with the negative electrode, the blade of the leaf in contact with the positive electrode, the epicotyl tip and adjacent leaf petioles, and the roots and stem. In the control plants one of the primary leaves of each was arbitrarily designated as leaf 1 and the other as leaf 2 (Table 1), as control comparisons for the leaves attached to the anode and cathode, respectively, described above. The dried plant tissue samples were assayed for radioactivity using an end window G-M tube and standard scaler circuit. Autoradiograms were prepared as described by Wittwer and Lundahl (1951).

The apparatus for supplying electric currents consisted of a voltage stabilizer (Solavolt, Fischer Scientific Co., Chicago) connected to a step-up transformer and a rectifier circuit. This circuit was capable of producing 40 milli-amperes at 250 volts using several 0.025 to 0.10 resistors (Ohmite Manufacturing Co., Skokie, Ill.), and pencil lead (graphite) resistance circuits lined out on the surface of Masonite insulating boards. The essential details of the methods employed with the plant as an electrophoretic unit may be found in reports by Olien (1956, 1961). Coarse adjustments in current flow were made by varying the number of resistors in the circuit and the thickness of the pencil lead lines on the Masonite insulating board. Fine adjustments were made by varying the voltage input to the rectifier circuit. This permitted a variation of from 200 to 240 volts across the plant tissue.

Results and Discussion

Results of several typical experiments are summarized in Table 1. The most striking effect of the electrical current in the plant was the preferential migration of Ca^{45} into the leaf to which the anode was attached. This selective transport toward the positive electrode was most pronounced and highly significant at both the 30 and 40 micro-ampere levels. Even at 16 micro-amperes almost twice as much Ca^{45} migrated to the leaf in contact with the

Table 1. *The effect of direct current on the transport of Ca^{45} from the epicotyl tip into the primary leaves of bean seedlings. The electrophoretic unit extended from the tip of one primary leaf to that of the other. — Per cent of Ca^{45} applied transported from epicotyls in 24 hours.*

Micro-amperes of current applied (7-hour duration)	Plants subject to direct currents		Plants not subject to direct currents	
	Leaf attached to anode	Leaf attached to cathode	Leaf 1	Leaf 2
16	37.3 ¹	19.9	28.8	34.0
30	43.1	7.6	27.0	23.1
40	48.3	6.7	20.8	24.7

¹ All values in this column significantly greater ($P=0.01$) than values for leaves exposed to the same amperage but attached to the cathode. Differences in leaves from plants not subject to direct currents were not significant.

positive electrode. There were no effects of electric currents on Ca^{45} transport or accumulation in any tissues other than the leaves. Calcium⁴⁵ levels in the two primary leaves of plants not exposed to direct currents (Table 1) showed the usual variations expected even in carefully selected biological materials. The percentage of the applied Ca^{45} that was transported from the leaf axils into the two primary leaves was approximately the same, however, for plants exposed and those not exposed to a given amount of direct current.

Autoradiograms (Figure 1) confirmed the results in Table 1. The transport of Ca^{45} from the leaf axils and epicotyl tip into the leaf in contact with the anode was consistently greater. Distribution within the leaf blades of the translocated Ca^{45} , as reflected by a predominance in the veins, was comparable to that in leaves attached to the cathode and leaves of control plants. The autodiagrams suggest that transport of Ca^{45} under the influence of direct current followed the usual vascular channels.

In other experiments Ca^{45} solutions were applied as droplets to the upper leaf blade surfaces of leaves in contact with both negative and positive electrodes. With the same electrophoretic plant unit as that described above no physiologically tolerable electrical current, however, resulted in the basipetal transport of detectable quantities of the isotope out of the leaf onto which it was originally deposited. Nor did the presence of direct currents in the plant tissues affect foliar absorption of Ca^{45} , either through the upper leaf surface or in that taken up by epicotyl tips and leaf axils.

It has not been established by these studies that the observed influence on calcium transport can be entirely attributed to a direct effect of electrical polarity. Injury to leaf tissue as evidenced by necrosis and general collapse of plant parts was largely avoided by using a current of less than 50 micro-



Figure 1. *Autoradiograms depicting the effect of direct current on the distribution of Ca^{45} in bean seedlings.* A droplet of $\text{Ca}^{45}\text{Cl}_2$ solution was applied in each instance to the epicotyl tip. A, non-treated (control) plant; B, C, and D, each plant consisted of an electrophoretic unit in which a negative contact was made to the tip of the primary leaf on the right, and a positive contact to the tip of the primary leaf on the left; B, a current of 40 micro-amperes, and C and D, 16 micro-amperes applied for 7 hours. All plants harvested 24 hours after application of the isotope and 17 hours after the electrical treatment was discontinued.

amperes, and an exposure of 7 hours or less. Injury was associated with the total amount of electrical energy applied. The critical amount was close to the 0.86 coulombs (10 micro-ampere days) reported by Olien (1956) for wheat plants. In the present experiments little if any visible tissue damage occurred at a current level of 16 micro-amperes for 7 hours. Where injury developed it was limited to a very small necrotic area at the fringe of the moistened carbon and on the tip of the negative leaf. At current levels of 30 and 40 micro-amperes the necrosis was more consistent and covered a larger area of the leaf tip. The petiole of the leaf attached to the anode developed a partially dehydrated appearance after the current had flowed for approximately 5 hours. The presence of some degree of injury to plant tissues associated with the enhancement of Ca^{45} transport into the leaf blade in contact with the anode cannot be precluded. Possible alterations in transpiration induced by leaf injury were not measured but may have contributed to the differences observed in Ca^{45} transport.

The nature of the preferential transport of Ca^{45} ions toward the positive

electrode in the above electrophoretic plant units is not known. Apparently the positively charged calcium ion is attached to a negative carrier which migrates toward the positive terminal. Such a carrier has not been identified.

Summary

Radiocalcium applied to the epicotyl tip of bean seedlings subjected to direct current was caused to preferentially migrate into the primary leaf blade to which the anode was attached. This was demonstrated by radio-analysis of dried plant tissues as well as by autoradiography. The electrophoretic plant unit consisted of the negative contact on the tip of one primary leaf and the positive contact on the tip of the opposite primary leaf with the plant tissue extending between. A direct current of 16 to 40 micro-amperes applied for 7 hours was effective in altering the transport of Ca^{45} . Some tissue injury, though very slight, was associated with current levels that greatly altered Ca^{45} transport.

Direct current across the plant tissue did not induce basipetal transport of Ca^{45} nor alter its rate of absorption by the foliage.

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Effects of Reduced Oxygen Tension on Vascular Plants

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The phenomena of oxygen toxicity, although by no means recent in their discovery, have excited the interest of physiologists only within the past few years (4, 7). The study of oxygen-induced damage has been carried out principally at extra-physiological oxygen tensions, but there is no reason on chemical grounds to view the effects obtained as unique and discontinuous with behavior at lower oxygen tensions. Further, it is known that specific processes, hemoglobin synthesis, for example, are inhibited by oxygen tensions in excess of about 50 mm. (3); that cucumber hypocotyl elongation can be markedly enhanced by preconditioning the seedlings under argon (9); and that seed germination in various plant species may be favored by submergence. It has recently been shown that a number of Metazoans including molluscs and nematodes can survive, and perhaps flourish, under conditions of near or complete anaerobiosis (5).

The foregoing considerations, and others (2, 6, 8, 9) led us to question the real extent of oxygen dependency in organisms which, by convention are regarded as aerobes (1). Experimentally, a thorough study must include the use of oxygen tensions spanning the range $pO_2=0-150$ mm. Hg. The present communication deals only with preliminary studies carried out at extremely low pO_2 levels.

The experimental environment was provided by a rectangular plexiglass chamber about 3 cu. ft. in volume which was flushed continuously with watersaturated argon. Chamber temperatures ranged from 25–28°C. At the inception of any experiment the chamber was flushed for 3 hrs. at 30 cu. ft./hr., the flow rate thereafter being

reduced to 6–8 cu. ft./hr. The chamber was not completely gastight, hence average oxygen concentration in its atmosphere was estimated by the use of alkaline pyrogallol solutions, the oxidation (and coloration) of which are proportional to pO_2 and can be followed photometrically. Supplementary measurements were made using a sensitive coupled glucose oxidase-peroxidase system in which the rate of pyrogallol oxidation was proportional to H_2O_2 generated. The details of these systems will be described elsewhere, but relative to air, the chamber atmospheres supported rates of oxidation corresponding to oxygen concentrations of 1 % or less. The CO_2 level has not been measured as yet, however, the continuous flush system employed suggests that it must be relatively low.

Survival studies were carried out using 3–5 week old *Salvia*, tomato, *Alyssum*, *Coleus* and *Chrysanthemum* seedlings or cuttings, 10 day old *Acer saccharinum* seedlings, and occasional weed seedlings growing with cultivated species (*Digitaria*, *Oxalis*, *Plantago* for example). These plants were maintained in potting soil.

Survival times were based upon irreversible wilting which was observed by the withdrawal of samples into air. Only minimum values were obtained in some instances, and the results for many species were based upon few plants, hence are highly approximate.

Alyssum, *Salvia*, and *Chrysanthemum* survived for more than 24 hrs; *Digitaria*, *Oxalis*, and *Plantago*, survived more than 40 hrs. The maximum survival time for *Coleus* lay between 150 and 175 hrs., and for *Acer*, between 250 and 275 hrs. In contrast, tomato plants could not tolerate as much as 25 hrs. under reduced oxygen tension. The survival of *Coleus* and *Acer* were increased markedly when the plants were watered with 0.005 *M* KNO_3 . *Coleus* tolerated an additional 100 hrs. and the maple, an additional 25 to 40 hrs.

Seed germination was also modified by chemical substances, but proceeded well with water alone. Germination tests were carried out with groups of 50 seeds on filter paper in petri dishes. After 5 days, winter rye had germinated 37 % (3 trials). In the presence of adenosine triphosphate (ATP) or KNO_3 (0.005 *M*) about 55 % germination (3 trials) was observed. Root and coleoptile emergence of as much as 3–5 mm. was noted, both in water and test solutions. In 4 days Cucumber germinated about 20 % in water and 40–45 % in KNO_3 solution. Maple seeds had failed to germinate after 10 days in the argon chamber, but retained their viability, germinating 80 % after being returned for 8 days to air.

These studies reveal a wide range of responses to reduced pO_2 and their modification by metabolically important substances. The most striking behavior was exhibited by *Lemna*, however. When this species was grown in Hillman's medium (culture and medium supplied by Dr. R. M. Klein, New York Botanical Gardens), which contains sucrose as a carbon source, the frond count during the first 10 days increased at one-twelfth the aerobic

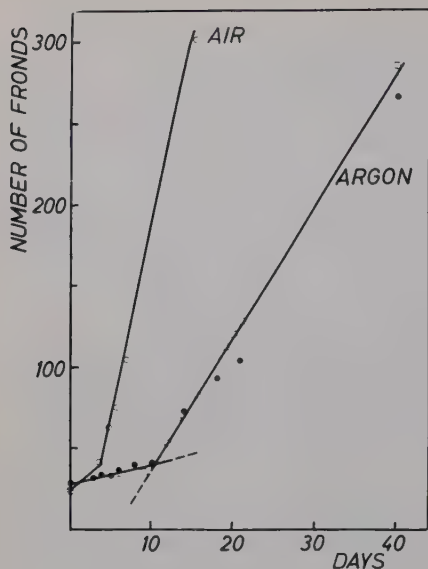


Figure 1. The growth of *Lemna* in air and low oxygen tension. Open circles, air control; solid circles and squares, two experiments at low pO_2 .

rate. During the subsequent 30 days, the growth rate in two experiments at low pO_2 was approximately one-half the initial aerobic rate (Figure 1). Neither the rate nor the induction period were affected by daylight fluorescent light at 75–100 foot-candles.

These experiments show that the vascular plant, like the Metazoans mentioned and many simpler organisms, retain considerable potentialities for existence as microphilic aerobes and perhaps facultative anaerobes. It seems reasonable to suggest that many plant and animal forms possess heretofore unsuspected tolerance to reduced pO_2 and perhaps optimal oxygen levels well below that of air.

It remains to be determined whether or not there exist levels of oxygen which allow growth and development to proceed in the absence of effects involving oxygen damage.

Summary

Recent studies of O_2 toxicity, antioxidants and anaerobiosis in various organisms suggested the value of experiments on the O_2 dependency of vascular plants. A number of species tolerate 24 to more than 250 hrs. in argon containing $< 1\%$ O_2 . *Coleus* and maple were among the more tolerant species and showed increased survival when KNO_3 was supplied. Both KNO_3

and ATP stimulated germination of Rye grains and Cucumber seeds. Lemna shows adaptation to low pO_2 , initiating growth at an appreciable rate after a 10 day lag period.

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Photoinactivations and Their Reversals in Growth and Motility of the Green Alga *Platymonas* (Volvocales)

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Introduction

When biological material is irradiated in the far ultraviolet region below 300 m μ different kinds of inactivations may occur. Between 240 and 300 m μ an inactivation is effected which by action spectra determinations and biochemical analyses has been shown to be due to damage to nucleic acid metabolism. Reviews on photoinactivation and photoreactivation have been given by Dulbecco (1955), Jagger (1958), Shugar and Wierzchowski (1958), Kleczkowski (1960), and the action spectra have in particular been dealt with by Setlow (1957). This inactivation is often delayed and in many cases it is first detectable after one or two cell divisions (Stuy 1959 and the present publication). Stuy (*loc. cit.*) also reported that a decrease in the growth curve of ultraviolet treated (254 m μ) *Haemophilus influenzae* occurred after 80 minutes, and that this break coincided with a marked decrease in the deoxyribonucleic acid (DNA) content. This block in DNA synthesis after irradiation with far ultraviolet was first shown by Kelner (1953) and has later been demonstrated by several others (Kanazir and Errera 1956, Iverson and Giese 1957, and Halvorson and Jackson 1956). Kelner, Halvorson and Jackson, and Iverson and Giese further demonstrated that certain doses of far ultraviolet radiation could abolish DNA synthesis, whereas ribonucleic acid (RNA) and protein synthesis were permitted to continue for some time.

As a rule the damage to the nucleic acid can partly be remedied by near ultraviolet radiation and radiation in the violet and blue region of the visible

spectrum. This repair is called photoreactivation. A few hours in the dark before this after-irradiation will prevent the reversal. A faint photoreactivation has also been demonstrated with damages by radiation of wave lengths shorter than 200 m μ and with X-rays but these examples are exceptions (for literature see Jagger 1958).

The photoreactivable injuries to biological systems caused by irradiation with far ultraviolet have thus almost without exception been traced down to nucleic acid metabolism. On the other hand, very little is known about the chromophore which is involved in the recovery process. Experiments with enucleated fractions of amoeba and frog and crab nerves have demonstrated that the chromophore is situated in the cytoplasm, but the possibility that it also is present in the nucleus has not been excluded (Skreb and Errera 1957, Pierce and Giese 1957). The action spectra analyses of photoreactivation that have been performed have in many cases given extremely different results.

The bacteriophage T2 adsorbed on *Escherichia coli* showed a region of high effect between 313 and 400 m μ both for photorecovery of the phage and the bacteria. Small maxima occurred at 324, 350, and 385 m μ , no effect was obtained above 500 m μ (Jagger and Latarjet 1956). The action spectrum for photoreactivation in *Streptomyces griseus* had a very clear maximum at 430 m μ . No effect was shown above 500 m μ and no readings were made below 365 m μ (Kelner 1951). Giese *et al.* (1952) studied the photoreactivation of the division delay injured by 254 m μ radiation, by the use of mercury spectral lines, in the ciliate *Colpidium colpoda* and recorded maximum effect at 365 and 436 m μ . No photoreactivation was observed at longer wave lengths. Recently Tageeva and Dubrov (1961) studied the photoreactivation in epidermis of onion scales *Allium cepa* and their action spectrum showed a distinct maximum around 430—450 m μ . Tageeva and Dubrov assumed that a flavin system is involved and the results of Giese *et al.* (*loc. cit.*) suggest the same. As a whole the different action spectra analyses that have been performed on photoreactivation do not point to a single chromophore type which is universally involved in the recovery process. The possibility that different chromophores are involved seems more probable.

Jagger (1958) paid attention to the energy content of the quanta in different regions of the spectrum and to what degree this factor might influence the action spectra determinations of photoreactivation. Radiation below 300 m μ causes damage to proteins and nucleic acids by loosening the weak hydrogen-bonding of these large molecules and as a rule inactivations of biological processes occur at these wave lengths. Phototropism has, however, been demonstrated in this spectral region at low intensities (Curry and Gruen 1959, Delbrück and Shropshire 1960) and also phototaxis (Halldal 1961).

In the latter case it was shown that radiant energy absorbed by a protein molecule was effective, possibly through an energy transfer to a carotenoid. With the energy doses that are needed to effect photoreactivation, inactivations most certainly result below 300 m μ and this makes an action spectrum analysis for photoreactivation impossible at these wave lengths. One of Jagger's (1958) assumptions that the energy content of the quanta around 450–500 m μ suddenly might become too small to effect photoreactivation seems less probable. This is dealt with in the discussion.

Brandt and Giese (1956) observed that low intensity radiation between 226 and 239 m μ rapidly immobilized the ciliate *Paramecium caudatum*. This immobilization was not photoreversible but the ciliates resumed motility up to 80 per cent after the 226 m μ radiation, and less at longer wave lengths. The action spectrum curve for this immobilization resembled the absorption curve of proteins and a damage to the cytoplasm was assumed. A similar autoreversible immobilization of *Platymonas* will be described in the present publication.

Photoreactivation has mainly been studied in viruses, bacteriophages, bacteria, and protozoa. A short report has been given on the photoreactivation in the growth of the unicellular alga *Chlamydomonas moewusii* by Weis (1952); and Lyman *et al.* (1959), and Schiff and Epstein (1961) studied photoreactivation in the development of *Euglena* chloroplasts.

The present investigation has been concerned with the different types of inactivation and their reversals in the unicellular alga *Platymonas subcordiformis*, which now for some years has been used in experiments on phototaxis. Attention has in particular been paid to action spectra analyses with the hope that these will give more information about the chromophore involved in photoreactivation.

Material

The unicellular Volvocales *Platymonas subcordiformis* (Wille) Hazen (Gibor's strain) has been used in these experiments. The alga was grown in a synthetic medium (Halldal 1960) in 2-litre Erlenmeyer flasks in a growth cabinet maintained at 21°C. The light intensity was about 3,000 Lux which was produced by four 90-W General Electric fluorescent tubes (daylight type) with one 40-W incandescent lamp to support some additional red light. As samples for the experiments had to be collected at different times during the day continuous illumination was used in order to assure uniformity in the population. A transfer to a new medium was performed every other day from a 10- to 12-day old culture, and cells from 10- to 12-day old cultures were used for experiments.

Provided that proper pH and ion composition in the medium are maintained, the motility of a population is a good indication of what may be called the general

physiological condition of the algae. Two days after the inoculation in a new medium a population is obtained which has about 80 per cent of the cells in motion. This situation is unchanged for about two weeks. Together with the changes in the growth of the population deviations from these 80 per cent have been used as a tool in studies of the effect of different radiations. It will be shown later that analyses of the motility are much more sensitive than the measurements of changes in population density.

Methods and Apparatus

Inactivation at 254 m μ . Inactivation at this wave length was effected with a Westinghouse WL 782.30 sterilamp. 100 ml. of the culture was centrifuged for five minutes at about 500 *g* in order to concentrate the population about four times. 5 ml. of this was then transferred to a shallow watch glass, 10 cm. in diameter, and this sample was irradiated at 20 cm. distance from the lamp. The radiant energy falling upon the sample was measured with a solar cell (an unprotected flat surface p-n junction in a GaAs crystal with surface area about 32 mm² prepared by Dr. R. Gremmelmaier, Erlangen, Germany) in combination with a Siemens Nanoampèremeter.

The Institute of Biophysics, University of Copenhagen, Denmark made available a RCA 1P 28 photomultiplier tube which was calibrated in relative sensitivity over the spectrum. The 1P 28 tube used during the present investigation was checked against this. At 223 m μ , at the cut-off side of the sensitivity curve for this type of tubes, the deviation was 10 per cent from the curve published by the manufacturer. At longer wave lengths the deviation was insignificant. This is in agreement with Engstrom's statement about the stability in spectral sensitivity of this type of tubes (Engstrom 1947).

The photomultiplier tube was then calibrated in absolute energy through a Moll & Burger Standard Thermopile (E5), firm Kipp & Zonen in combination with a Kipp & Zonen Portable Galvanometer (Pa A 70) which was calibrated for voltage deflection, by the use of the strong mercury spectral line at 365 m μ isolated by a Bausch & Lomb 250 mm. grating monochromator.

By the use of the calibrated 1P 28 photomultiplier tube and the solar cell the radiant intensity on the sample under the sterilamp was calculated to be 9,000 erg/cm²sec.

Action spectra of inactivation. For these experiments a Bausch & Lomb grating monochromator with 600 grooves/mm. was used in combination with a commercial Hanovia quartz sun lamp which had high energy output in far ultraviolet. The mercury lines between 223 and 313 m μ were isolated with the slit adjusted to give a spectral band width of 7 m μ . The full energy output at different wave lengths was used during irradiation. The energy output was measured with the 1P 28 photomultiplier tube mentioned above.

As ethylene-diamine-tetra-acetic acid (EDTA-Na) which was used as a chelator, and tris(hydroxymethyl)aminomethane (Tris) which was used as a buffer in the medium absorb significantly below 300 m μ , irradiation of cells in the culture medium could not be carried out in action spectra analyses. The cells were therefore centrifuged down and resuspended in the following solution: NaCl, 0.5 *M*; CaCl₂, 0.01 *M*; MgCl₂, 0.02 *M*; KCl, 0.006 *M*; KHCO₃, 0.001 *M*. The pH of this mixture was 7.5.

In this solution the algae maintained an optimal motility for several days. One milliliter of this suspension was transferred to a crystal quartz cuvette and the sample irradiated at the image of the grating. At zero time irradiation 0.1 ml. was removed and from then on every 15 minutes for one and a half hour a similar sample was taken and transferred to 5 ml. of culture medium in culture tubes with glass covers and immediately placed in the dark where they were kept for at least 12 hours. They were then allowed to grow and develop in a growth cabinet which contained light in the spectral region between 500 and 800 m μ for one week when the readings were made. At the same time as the transfer to the culture tubes a hanging drop sample was taken for microscopical studies of immediate reduction in motility. A small drop was transferred to the bottom of a container consisting of a glass ring fastened to a microscope slide. This container was inverted and the hanging drop was illuminated from the side and studied under the microscope. The cells in motion and not in motion were recorded in rectangular areas, 1.70 \times 0.85 mm. marked by threads in the eye-piece, nearest the light source, farthest away from it, and at the right and left side of the drop. The per cent in motion was then calculated. In determining the action spectrum of immediate reduction in motility the reciprocals of the incident numbers of quanta which caused a 20 per cent reduction in the motility from the original value of around 80 per cent (accurately determined in each case) were plotted against wave lengths of radiation. The action spectrum of delayed reduction in motility was determined by plotting the reciprocals of the number of quanta that caused a 50 per cent reduction after one week.

Photoreactivation in near ultraviolet and "white" light. These experiments were of a preliminary nature and were performed in order to study to what extent photoreactivation occurred in *Platymonas* and whether the growth or the motility was the best tool in action spectra analyses. *Platymonas* samples were inactivated under the sterilamp in the usual way (see above) for 0, 4, 8, and 16 minutes which exposed them to energy up to 8×10^6 erg/cm 2 . From these irradiated samples 0.3 ml. was removed and transferred to 10 ml. medium in culture tubes with glass covers. These were then placed in a glass beaker which was slowly rotating by a motor at one turn every minute, where the sample was after-irradiated with a Philips HPL 125-W mercury lamp with frosted bulb in an optical system which focused the image of the lamp upon the sample. Infrared radiation was removed by 1 cm. 10 g. CuSO $_4 \cdot 5$ H $_2$ O/l. The intensity at the sample as measured with the Thermopile was 3,200 erg/cm 2 sec. After-irradiation was effected for 0, 30, and 60 minutes for each of the samples given different doses of inactivation radiation.

The changes in the population density were studied with an Evans Electro-selenium Nephelometer. The galvanometer scale of the nephelometer was calibrated in absolute values for cell numbers by reading the deflections from cultures with known cell density as determined by a hemacytometer. Zero readings were taken for each culture tube with medium and no algae. The motility was studied as described above. After the last irradiation the samples were immediately placed in the dark for at least 12 hours and from then on in the growth cabinet with the longer wave lengths light described below. Cell density and motility were recorded one day after the irradiation and from then on every other day for ten days. The experiment was performed with four parallels and it has been repeated twice.

Action spectrum of photoreactivation. For these experiments the algae were inactivated under the sterilamp for 5 minutes. In most cases this dose produced a population which on the seventh day had between 7 and 10 per cent of the cells

in motion. In each case the per cent in motion was determined accurately. The effect of the different wave lengths in photoreactivation could then be determined by plotting the per cent of cells in motion against dose of the longer wave length radiation. The action spectrum of photoreactivation was then determined by plotting the reciprocal of the incident numbers of quanta that caused a standard degree of reactivation against wave lengths of radiation. This standard degree of reactivation was selected from a population which contained 20 per cent of cells in motion added to the percentage in the not after-irradiated sample. This method could be applied at all wave lengths except at 480 m μ where extrapolation had to be made because of the low effect. The curve presented in Figure 5 is based upon such measurements. In addition to this the relative heights of the curve at 308, 365, 382, 405, 436, and 480 m μ have been carefully checked and confirmed by changes in the inactivating dose at 254 m μ , followed by reactivation at different wave lengths.

For experiments on photoreactivation spectral bands were isolated from a water cooled Philips 500-W super high pressure mercury lamp by means of interference filters. The mercury lamp was run from a Philips Voltage Stabilizer (Type PE 4222). The reflecting mirror of the lamp was removed and the arc was projected at a distance of 1.4 m. by means of a quartz lens. This beam entered a light-tight box through a window where the interference filters and additional Schott filters were placed. The quartz lens and the filters were cooled by air streams directed to the region between the lens and the filters, and the lamp. In this way a diverging fan-shaped beam with reasonably good spectral purity was produced (see Table 1). The maximum angle of the beam from the normal of the filters was 8°, which is within the tolerance for the use of interference filters. The beam was reflected by means of a surface aluminized mirror onto the bottom of a crystal quartz cuvette with optically polished bottom. The cuvette was fastened to a motor and rotated at two revolutions per second. The radiant energy was measured with a Moll & Burger Thermopile (see above), and the intensity adjusted by changing the distance between the lamp and the sample. A fixed radiant energy of 6,800 erg/cm²sec. was applied at different wave lengths for two hours. At 480 and 506 m μ this high intensity could not be obtained. In these cases the exposure time was increased to give the same dose of radiation. In the ultraviolet the measurements were made possible by the use of five Jena Schott and Gen. interference filters of high quality which the Institute of Biophysics, University of Copenhagen, Denmark made available for these experiments. In the visible region Balzers (Liechtenstein) interference filters were used in combination with Schott glass filters. The filter combinations used and some filter characteristics are given in Table 1.

After the irradiation under the sterilamp 1.5 ml. of the algal suspension was transferred to the quartz cuvette and a sample of 0.15 ml. was removed by a sterile glass tube connected to an adjusted syringe and this sample was transferred to a culture tube with 5 ml. medium in the usual way. From this time on a similar sample was removed from the algae under after-irradiation every 15 minutes for two hours. The growth and the recording of the motility were performed in the usual way.

Treatments after the irradiations. For the growth and development of the algae it was necessary to use light that was effective in photosynthesis but ineffective in photoreactivation. There are only two reports in the literature which state that light of wave lengths longer than 500 m μ has caused photoreactivation, namely that of Giese *et al.* (1953) who observed some photoreactivation in starved *Colpidium col-*

Table 1. Filter combinations used in action spectra analyses of photoreactivation.

Interference filters		Schott filters 2 mm.	1 cm CuSO ₄ · 5H ₂ O 10 g/l.
mμ	HW mμ		
Jena Schott & Gen.	308	6	
	330	7	
	353	7.5	
	364	9	
	382	14	
Balzers	405	10	
	427	12	BG 12
	480	20	BG 12
	506	20	GG 9
	542	20	GG 11
	(578)		GG 14
		OG 2	+
			+
			+
			(+)

poda with the mercury spectral line at 546 mμ, and that of Cantelmo (1951) who reported that the greatest effect was obtained at 660 mμ for the phage development in *Staphylococcus aureus*. Cantelmo's results have been questioned by Dulbecco and Weigle (1952), and by Jagger (1958). The growth cabinets were therefore constructed with the assumption that light of longer wave length than 500 mμ was neglectably effective in photoreactivation. This was later confirmed by action spectra analyses of photoreactivation. As light source was used a Delka 150-W projector spot lamp. The light from this lamp was first filtered through 1 cm. plexiglass which removed the infrared radiation above 2 μ, then through 6 cm. of water with some CuSO₄ in a plexiglass box which removed the rest of the infrared, and finally through two layers of yellow celluloid which removed all radiation at wave lengths below 500 mμ. The celluloid filter was the top of a box where the culture tubes with the algae were kept. Air spaces were kept between these different filters, and the box and the filters were cooled by fans. The temperature within the box was between 23 and 25°C. The samples were given 12 hours light and 12 hours dark. The radiant intensity within the box was 14,400 erg/cm²sec. This corresponded to 1,000 Lux as measured with a Photovolt Corporation, model no 200 A, foot candles meter.

The algae in the box were thus exposed to light at wave lengths between 500 and 800 mμ which should be ineffective in photoreactivation. In order to eliminate a possible faint photoreactivating effect at these wave lengths the samples were kept in the dark for at least 12 hours after the last exposure. Photoreactivation after such a period of time is difficult to demonstrate. The culture tubes were placed at random in plastic stands in the box and thereafter randomized every day.

Results

Photoinactivations

Action spectrum of immediate reduction in motility. The action spectrum is given in Figure 1. It is shown that the 223 mμ radiation is extremely effec-

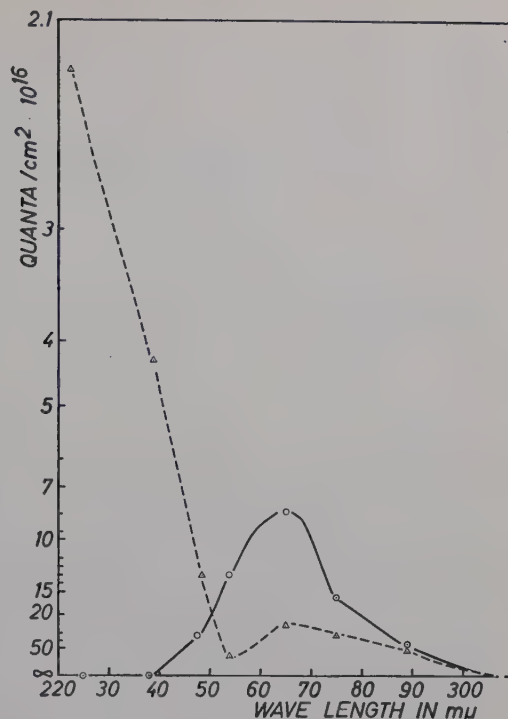


Figure 1. Action spectra of two types of immobilization in *Platymonas* recorded as reduction in per cent of cells in motion. - - - -: Immediate reduction in motility to 20 per cent of original value; ———: Delayed reduction in motility to 50 per cent of original value.

The ordinate is linear for $\frac{1}{n}$ where n denotes the numbers of quanta marked along the axis.

tive and all cells are usually immobilized after 10^{17} quanta/cm². The effect of the 238 mμ radiation is definitely lower and at longer wave lengths a reduction in motility is first observable after prolonged exposure time. The action spectrum to 95 per cent immobilization has also been determined and this curve showed the same details as those in Figure 1, but due to the inconveniently long exposure times (up to 7 hours at 254 and above 275 mμ) this method was abandoned. The action spectrum curve shows a minimum at 254 mμ, a maximum at 265, and from this wave length a decrease was observed and no effect was obtained above 300 mμ. This corresponds fairly well to the absorption of proteins. Approaching 300 mμ from the shorter wave length side the effect of the quanta in denaturing the proteins is constantly reduced (McLaren 1949). As a rule a general decrease occurs in the quantum yield from shorter to longer wave lengths except in the region around 250 to 260 mμ where a maximum usually was observed (Setlow and Doyle 1957). Distortions in action spectra curves of inactivation of proteins similar to that presented in Figure 1 were also demonstrated by Setlow and Doyle (*loc. cit.*) and in Setlow's review of 1957. The lack of coincidence between a typical protein absorption curve and the action spectrum of imme-

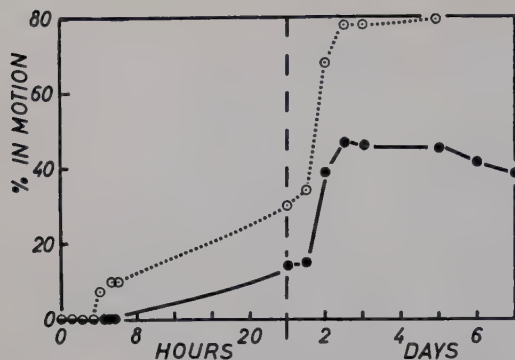


Figure 2. The recovery in motility of a *Platymonas* population which was immobilized with a dose of 10^{17} quanta/cm² at 223 m μ: Recovery in light between 500 and 800 m μ ; —: Recovery in the dark.

mediate reduction in motility seems therefore to be due to difference in damaging effect at different wave lengths, and the curve most reasonably stands for protein absorption. A similar curve was also reported for the immobilization of cilia movement in *Paramecium caudatum* (Brandt and Giese 1956).

The immobilization of *Platymonas* at 223 and 238 m μ is autoreversible. This is shown in Figure 2. In this experiment a *Platymonas* population was exposed to 10^{17} quanta/cm² at 223 m μ . No motion was observed after 7×10^{16} quanta/cm² but in order to ensure complete immobilization the population was given an additional dose of 3×10^{16} quanta/cm². Microscopical examination showed that the flagella were intact on the cells after such exposure. The sample was then divided and each half was transferred to 5 ml. of medium in culture tubes. One of the tubes was placed in the dark, and the other in the growth cabinet with light between 500 and 800 m μ . Samples for examination of the motility were removed every half hour the first day and later on once a day. After 4 hours in the light some cells started to move (motility 7 per cent) and from then on a steady increase occurred and full motility was resumed on the second day. In the dark the recovery was much slower, and it was never complete. No motion was observed during the first 6 hours. On the next day 15 per cent were in motion and the maximum occurred on the second day with 46 per cent. From then on a steady decrease took place. A control sample which had not been irradiated and was kept in the dark showed no reduction in motility during the first seven days.

Action spectrum of delayed reduction in motility. This action spectrum is shown in Figure 1. No effect was found at 223 and 238 m μ . Between 248 and 289 m μ a clear effect was obtained with maximum at 265 m μ . This action spectrum corresponds closely to the absorption spectrum of nucleic acid (see Mallette and Lamanna 1954). A damage to DNA anabolism is most probably involved at these wave lengths.

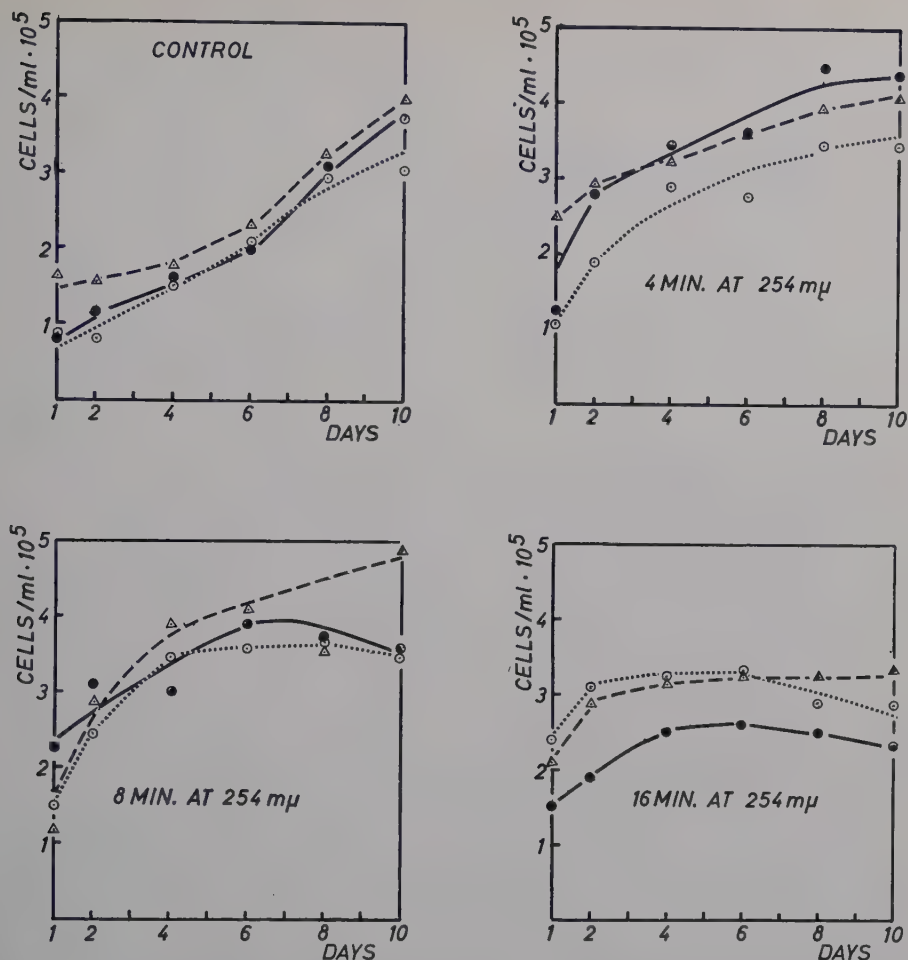


Figure 3. Photoinactivation and photoreactivation of *Platymonas* populations recorded as changes in cell numbers. The doses of the inactivating irradiations are given in the figure. Doses of reactivating near ultraviolet and "white" light: ——— not after-irradiated; after-irradiated with 5.75×10^6 erg/cm²; - - - - after-irradiated with 11.5×10^6 erg/cm².

Photoreactivation

Near ultraviolet and "white" light. The results from these experiments are given in Figure 3 for the growth and in Figure 4 for the motility. The growth curve for the control shows that a steady increase occurred in the population for at least 10 days with an increase in cell density from about 10^5 to about 4×10^5 per ml. which indicates two cell divisions. Also for the populations which were given a radiation dose at 254 mμ of 2×10^6 erg/cm² (4 minutes)

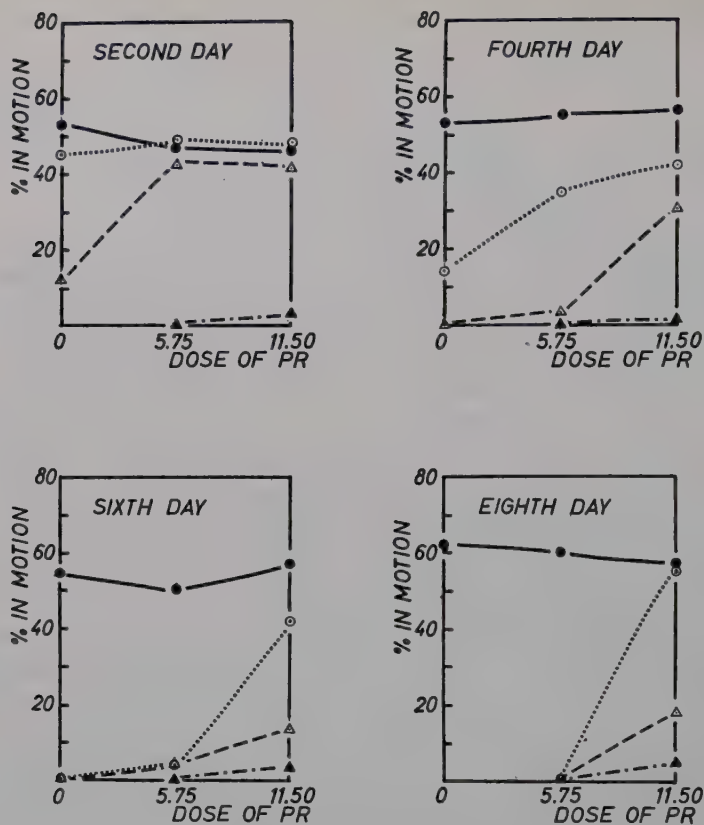


Figure 4. Photoinactivation and photoreactivation in *Platymonas* populations recorded as changes in the per cent of cells in motion. The dose of the photoreactivating irradiation (PR) with near ultraviolet and "white" light are given in the figure in $\text{erg/cm}^2 \times 10^6$. The dose of the 254 m μ irradiation: — not irradiated (control); irradiated for 4 minutes ($2 \times 10^6 \text{ erg/cm}^2$); - - - - - irradiated for 8 minutes ($4 \times 10^6 \text{ erg/cm}^2$); - · - · - irradiated for 16 minutes ($8 \times 10^6 \text{ erg/cm}^2$).

a steady increase occurred, but this increase was less than in the control and there was no significant effect of the after-irradiation. In the populations that were given a 254 m μ dose of $4 \times 10^6 \text{ erg/cm}^2$ (8 minutes) the effect of the after-irradiation is visible. With no after-irradiation and 30 minutes after-irradiation the growth stopped on the sixth day, but the growth increased in the population which was given 60 minutes of near ultraviolet and "white" light. This situation was about the same in the populations which were given 16 minutes of the 254 m μ radiation but the effect of the 60 minutes after-irradiation was very much reduced.

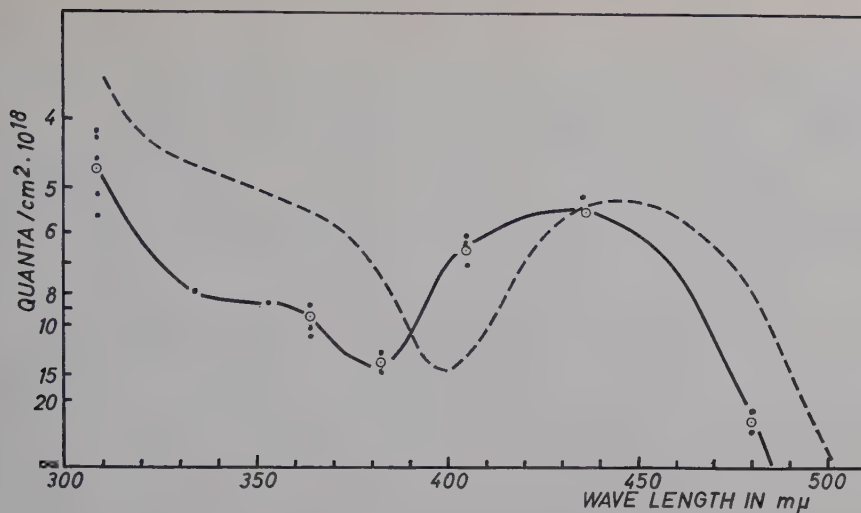


Figure 5. Action spectrum of photoreactivation in motility of *Platymonas* (—). The absorption spectrum of milk flavoprotein (---) given as $\log \frac{I_0}{I}$, in relative values according to Corran *et al.* (1939). The ordinate is linear for $\frac{1}{n}$ where n denotes the numbers of quanta marked along the axis.

The effect of the different 254 mμ doses and the after-irradiation at longer wave lengths was more clearly demonstrated in the analyses of the motility (Figure 4). On the second day no difference occurred in the control and in the population which was given 4 minutes exposure under the sterilamp, while a clear reduction in motility and also photoreactivation of the motility occurred in the populations which were given 8 and 16 minutes exposure at 254 mμ. On the fourth day the populations which were exposed to the 254 mμ radiation for 4 minutes also showed reduced motility and a clear photoreactivation, and this picture was in principle unchanged for the next four days. On the eighth day no motion occurred in the samples which were not after-irradiated (except for in the control) or after-irradiated for 30 minutes. 60 minutes of after-irradiation caused motility, and the percentage of cells in motion increased with decreasing 254 mμ dose. This is a demonstration that photoreactivation occurs both in growth and motility of *Platymonas* and that growth and motility are correlated in these experiments. It is finally shown that the motility is a much more sensitive method of recording photoreactivation. It must be emphasized, however, that different chromophores may be involved in the photoreactivation processes of growth and motility and that the action spectrum presented in Figure 5 does not necessarily stand for both, though this seems most probable.

The percentage of cells in motion is in these experiments somewhat lower than usual (50—60 per cent against the usual 80). This was due to a lower light intensity in the growth cabinets during these preliminary analyses. For this reason the radiant intensity was increased in later experiments.

Action spectrum of photoreactivation. The action spectrum is given in Figure 5. Photoreactivation occurred between 308 and 480 m μ . Below 300 m μ inactivations occurred (see Figure 1), and above 480 m μ no effect was found. Photoreactivation in the motility of *Platymonas* was thus confined to the spectral region where photoreactivation in general is demonstrated. The highest effect was observed at 308 m μ . From this wave length a rapid decrease occurred to a small shoulder at 365 m μ , a minimum at 382. From this wave length the effect increased to a maximum at 436 m μ . A small effect was observed at 480 m μ and no effect at the longer wave lengths tested (506, 546, and 578 m μ). The observations of Giese *et al.* (1952) on the ciliate *Colpidium colpoda* were too scarce to permit an action spectrum plot of photoreactivation, but their results indicate a curve similar to that obtained for *Platymonas* between 334 and 436 m μ . The same holds true for the results of Tageeva and Dubrov (1961) and Kelner (1951) for *Streptomyces griseus*, while the results of Jagger and Latarjet (1956), and Kelner's results from *Escherichia coli* (*loc. cit.*) are quite different. The action spectrum of photoreactivation in *Platymonas* resembles very much the absorption spectrum of a flavoprotein, which has been included in Figure 5.

Discussion

The results from the action spectra determinations for reduction in motility have much in common with the results of Brandt and Giese (1956) and Giese *et al.* (1952) for immobilization and delayed cell division in ciliates. The action spectrum for immediate reduction in motility of *Platymonas* coincided well with the action spectrum of immobilization of *Paramecium* and the curves indicate a damage of proteins (Figure 1). When *Platymonas* populations were immobilized in the spectral region where DNA absorbs insignificantly (223 and 238 m μ) this immobilization was autoreversible, to complete recovery in the light at wave lengths between 500 and 800 m μ , and to 60 per cent in the dark (Figure 2). Brandt and Giese (1956) also reported up to 80 per cent recovery from immobilization at these wave lengths. The light effect of the recovery in *Platymonas* is a photoreactivation according to the definition given by Jagger (1958): "Photoreactivation is the restoration of ultraviolet radiation lesions in a biological system with light of wave length longer than that of the damaging radiation". A closer examination of this

increased recovery in the light shows that this is not a true photoreactivation but a result of metabolism in general. The great energy content of the quanta around 223 m μ evidently inactivates the proteins, but leaves DNA and RNA, which absorb insignificantly at these wave lengths (Mallette and Lamanna 1954) intact. A resynthesis of enzymes can therefore take place. This is, however, dependent upon anabolism and in photosynthesizing organisms light is needed. The partial recovery in the dark indicates some heterotrophic growth.

The experiments which have been performed on photoreactivation have been largely concentrated upon non-photosynthetic material, and as a method of analysis cell division or growth has been employed. When performing experiments on photoreactivation in photosynthesizing material, the definition of Jagger should be extended to ensure at least one cell division under light conditions not involved in photoreactivation (as a rule between 500 and 750 m μ) before readings are taken.

The action spectrum for delayed reduction in motility (Figure 1) gave a curve similar to those for retardation of cell division in the ciliates, the inactivation-curves usually found for bacteriophages, viruses, and bacteria, and a damage caused by absorption of DNA is most probably involved (see introduction). This indicates that DNA, and not a nucleoprotein, is involved. Shugar and Wierzchowski (1958) emphasize the great difficulties that arise when action spectra of inactivation are to be interpreted, as the absorption curves of nucleic acids and nucleoproteins are nearly indistinguishable. The extinction coefficients of the nucleic acids at 260 m μ are about 30 to 60 times those of the proteins (Davidson 1953). Most of the radiation absorbed by a nucleoprotein will therefore be absorbed by the nucleic acid moiety. Zelle and Hollaender (1954) also showed by action spectra analyses that nucleic acid was the principal receptor in the inactivation of viruses, but the protein part was clearly reflected in their curve. Franklin *et al.* (1953) in their action spectra analyses also obtained evidence that the protein component also was involved. If a nucleoprotein were to be the radiant energy absorber in the delayed reduction in motility of *Platymonas*, this should most reasonably show up in the action spectrum by some increase in the action spectrum curve toward 200 m μ . The increase in quantum yield in protein destruction toward shorter wave lengths is then taken into consideration (McLaren 1949, Setlow 1954). This should compensate partly for the low absorption at these wave lengths. The ease with which proteins are damaged in living material is also demonstrated in the experiments of immediate immobilization in *Platymonas* (Figure 1) and *Paramecium* (Brandt and Giese 1956).

The delayed effect of this, most probably, DNA damage is clearly shown in Figures 3 and 4. This is in agreement with the observations that the

damage occurs to the DNA synthesis which, however, may continue for some time (Stuy 1959).

The reports in the literature about experiments in the far ultraviolet region below 300 m μ are concerned mainly with photoinactivations. This has led to the rather common misconception that only inactivations of biological materials occur at these wave lengths, which by no means is true. Both phototropism and phototaxis occur in this spectral region (Curry and Gruen 1959, Delbrück and Shropshire 1960, Halldal 1961), and *Platymonas* may respond phototactically to such radiations at intensities around 10^{10} quanta/cm² sec. for hours without the slightest effect upon the motility (unpublished results).

The action spectrum curve of photoreactivation has a definite maximum around 430 m μ (Figure 5). Due to the lack of observations between 430 and 480 m μ the peak could actually be situated anywhere between 430 and 450 m μ but probably not at longer wave length. A small minimum occurred at 385 and a small shoulder at 365 m μ . The highest effect was obtained by the 308 m μ radiation. These results strongly indicate that a flavoprotein is involved in the photoreactivation. In Figure 5 a flavoprotein absorption curve (Corran *et al.* 1939) is included for a comparison with the action spectrum. The 385 m μ minimum is at somewhat shorter wave length than the minimum in the absorption curve. This disagreement may, however, be due to internal screening of chloroplast pigments which will shift the minimum to a shorter wave length. This screening could also make the 430 m μ peak somewhat too low. The increase toward shorter wave lengths both in absorption and action spectrum curves is due to the pronounced 275 m μ absorption band of riboflavin. Proteins containing aromatic amino acids also have an absorption band around 280 m μ (see Takashima 1960 and Setlow and Doyle 1957), but the riboflavin band will be greatly dominating.

The action spectrum curve shows some resemblance to absorption curves of carotenoproteins. In the visible region the three absorption bands that are usually found in the carotenoids would not be resolved in the action spectrum measurements due to the scattered observation points in this region and the relatively broad spectral bands employed. The chief argument which eliminates a carotenoprotein as being the chromophore is the increase toward 308 m μ . Some increase in this spectral region could be expected due to the 280 m μ absorption band of most proteins. The increase in the action spectrum curve is, however, too pronounced to make this alternative reasonable.

The absorption characteristics of pterins show a reasonable resemblance to the action spectrum curve presented in Figure 5 (see Sinsheimer 1955). The maximum in the visible region occurs usually at a shorter wave length than the maximum in the action spectrum curve; the small details around 365 and

385 m μ are lacking, but both curves increase toward 300 m μ . The possibility that a pterin could be involved in photoreactivation can therefore not be completely excluded, but the action spectrum does fit the absorption of a flavoprotein much better.

The drop-off in the action spectra curves of photoreactivation around 450 m μ toward longer wave lengths most probably stands for a decreased absorption by the chromophore. Jagger's assumption (1958) that the energy content of the quanta in this spectral region suddenly might be too small to effect photoreactivation seems less reasonable, as the decrease in the effect coincides too well with the general decrease in absorption of pigments involved in biological processes (riboflavin, carotenoids, and pterins). Around 300 m μ it has actually been proved that a change occurs from activation to inactivation, not only because of the energy content of the quanta but also because a very particular type of molecules, namely the proteins and the nucleic acids start to absorb with some significance below 300 m μ . A transition from effect to no effect around 500 m μ caused by the drop in energy content of the quanta seems unreasonable.

Summary

Photoreactivation in both growth and motility of the green alga *Platymonas subcordiformis* was demonstrated. It was shown that the growth and the motility were correlated in this respect.

When irradiated in the far ultraviolet region between 223 and 300 m μ two types of inactivations occurred. One type was the immediate reduction in the per cent of cells in motion (immediate immobilization). The action spectrum of this followed the absorption curve of proteins. The other type, which was recorded after one week, gave an action spectrum which followed the absorption spectrum of deoxyribonucleic acid (DNA). At wave lengths where the absorption of DNA is insignificant (223 and 238 m μ) the immobilization of the cells was autoreversible, to complete recovery in the light at wavelengths between 500 and 800 m μ which did not affect photoreactivation, and to 60 per cent in the dark. It is concluded that the immediate immobilization is caused by destruction of proteins including the enzymes, and that these were re-synthesized during anabolism when the DNA synthesis was not damaged.

The action spectrum of photoreactivation showed great resemblance to a flavoprotein absorption curve. It is concluded that a flavoprotein is involved in photoreactivation of the motility, and most probably in the growth of *Platymonas*.

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Induction Phenomena in Photosynthesis of Algae

By

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1. Three types of Induction Curves

The CO₂-time curve of photosynthesis (uptake of carbon dioxide plotted as a function of time) is found to vary in shape during its initial phase, *i.e.*, from the onset of illumination until the assimilation processes have reached their maximum rate under the prevailing conditions (steady state photosynthesis). The shape of the curve during this induction phase depends on the identity of the sample material as well as on the experimental conditions chosen.

Previous investigations have shown the existence of three essentially different types of initial CO₂-time curves (induction curves):

Type 1, the primary-peak curve, is characterized by a steep increase, showing an intense uptake of carbon dioxide, immediately following the exposure to light. The increase continues for a period of about one minute, and is then followed by a decrease in the uptake rate for a shorter or longer period and subsequently by a secondary increase, with a slope of lesser steepness than the initial one. These changes in the rate of uptake result in a peak in the induction curve, generally called the primary peak or the one-minute peak. In addition to the primary peak several, but not all, time curves have one or more peaks occurring during later stages of the induction phase, so-called secondary peaks. Most probably the reactions responsible for the primary peak and for the secondary ones have no common cause (Aufdemgarten 1939 a, van der Veen 1949 a, Vejlby 1960 a). and in the pre-

sent paper interest is focused only on the primary peak and the cause of its appearance.

Primary-peak curves have been found in a vast number of plant species, including representatives of all major taxonomic groups ranging from algae to flowering plants (Harder and Aufdemgarten 1938, van der Veen 1949 a, b, 1950, 1960, Vejlby 1961, see also the survey in Table 1, p. 590 in this paper).

Type 2. In contrast to the primary-peak curve the second type of induction curve is remarkable by showing a brief output of carbon dioxide (a gush of carbon dioxide) immediately following exposure to light. After the conclusion of the gush, an uptake of carbon dioxide sets in and the time curve proceeds evenly, usually showing no tendency to peak formation until the level of steady state photosynthesis is reached.

We refer to time curves of this type as light-gush curves (antonym: dark-gush curves, see p. 591). They were first observed by Emerson and Lewis during experiments with *Chlorella pyrenoidosa* (1939). Subsequently, photosynthetic induction in certain other unicellular and multicellular green algae has been shown to follow a similar pattern (van der Veen 1950, Brown and Whittingham 1955, see also Table 1).

Type 3 represents CO_2 -time curves differing from both of the above types by showing an even curve with neither dips (gushes) nor peaks from the onset of illumination until the conclusion of the induction period. Hence this type of induction curve is characterized by a complete absence of the specific fluctuations known as induction phenomena.

We propose to introduce the designation half-arch curve for this third type. It was discovered by Aufdemgarten in experiments with *Coleus* leaves and in experiments with cells of the green alga *Stichococcus bacillaris* when the latter were grown in nutrient solutions poor in nitrogen (1939 b). It was further observed by Massini during investigations with a *Scenedesmus* strain (1957). More recently, the half-arch curve has been found in experiments with the moss species *Helodium blandowii* (Vejlby 1959 b).

2. Facts Concerning Light-gush Curves

Among the three types of curves representing the uptake of carbon dioxide during the induction phase, the primary-peak curve has been studied in the greatest detail by far. Vejlby has recently reviewed its historical data, its occurrence and distribution within the botanical system, and our present knowledge of the causes inducing the appearance of the peak (1961). The light-gush curve has not yet been studied in such detail, and the causes responsible for the existence of the gush are still obscure. However, several

interesting and valuable data have already been published (reviewed by van der Veen 1960). In the following we list a number of these observations which have been of particular interest when the present work was planned:

1. As far as we know, until now the light gush has been observed only in unicellular algae, and only in a minor number of species. These include *Chlorella pyrenoidosa* which was used by Emerson and Lewis when they investigated the phenomenon by means of the two vessel Warburg technique (1941). Emerson and Lewis' observations on the *Chlorella* gush were confirmed by van der Veen with the diaphragm method (1950) and by Brown and Whittingham with the mass spectrometer technique (1955). Further light-gush curves were found in experiments with *Protococcus olivaceus* (van der Veen 1950), and *Scenedesmus obliquus* (Brown and Whittingham 1955). A certain remark in the paper by Emerson and Lewis (p. 793) suggests that the list of species of algae producing light gush is somewhat more extensive than the one given here, but no more detailed information has been available. As a curiosity it should be noted here that Warburg in his many studies on photosynthesis in *Chlorella* (reviewed most recently by Bladergroen 1960) never observed any light gush.

2. The light gush does not appear in all strains of *Chlorella pyrenoidosa*. Hiller and Whittingham (1959) observed the phenomenon in only two strains out of 5, i.e., in one clone originating from Emerson's laboratory (the Emerson strain 3) and one clone originating from Tamiya's laboratory (the *Ellipsoidea* strain). Possibly the discrepancy between the results of Emerson and of Warburg as to the occurrence or non-occurrence of a light gush may be due to the fact that the latter author had only non-bursty strains at his disposal.

Apart from the observations of Hiller and Whittingham, it is noteworthy that McAlister and Myers (1941) failed to find a light gush in experiments with *Chlorella pyrenoidosa* in spite of the fact that their apparatus should easily reveal the existence of one. It would appear reasonable to assume that genetic factors were responsible for the negative result, as was the case in the study of Hiller and Whittingham. However, another important factor may rest in the cultivation method used by McAlister and Myers, atmospheric air being bubbled through the cell suspension in the stead of the carbon dioxide enriched air (3 to 10 % CO_2) used by all other workers in the field. In this connection it may be added that in an illustration (1957, Figure 4) of a *Chlorella* induction curve showing no light gush Gaffron states the determination to have been made with "air stream grown" cells.

3. As is the case with the reactions leading to the formation of the primary peak, the light gush is independent (in a limited sense) of the reactions responsible for the formation of oxygen in photosynthesis. O_2 -time curves do not show any peaks or depressions simultaneously with those appearing in the CO_2 curves. Hence the light gush and the primary peak are phenomena connected with the uptake of carbon dioxide exclusively (Emerson and Lewis 1941, Brown and Whittingham 1955).

4. The appearance and extent of the light gush depends on the length of the preceding dark period. The response is greatest after several hours of darkness (Emerson and Lewis 1941). When illumination is discontinued an uptake of carbon dioxide may be observed, filling a reservoir which in turn is emptied upon the onset of illumination.

5. The light gush phenomenon is not inhibited to any considerable extent by the addition of iodoacetamide (Hiller and Whittingham 1959). In this respect it

is similar to the reactions responsible for the appearance of the primary peak (Massini 1957, Vejlby 1959 c). On the other hand, iodoacetamide does affect one or more of the reactions responsible for carbon dioxide uptake or its use in the photosynthesis process proper, causing the rate of uptake to be considerably reduced and lowering the level for steady state photosynthesis.

6. A perusal of the literature shows that light gush and primary peak are never found together on one and the same induction curve. However, it appears to be possible for the two curve types, the primary-peak curve and light-gush curve, to occur in one and the same species, *i.e.*, *Chlorella pyrenoidosa*. Primary-peak curves were published for this species by McAlister and Myers in their paper of 1941, the time curve showing a distinct absence of any light gush. A strain of *Chlorella pyrenoidosa* from Emerson's laboratory was used for this study. No other cases of primary-peak curves appear to have been observed in *Chlorella* species.

The present work presents an attempt to check and supplement the classified observations concerning the occurrence of the primary peak and the light gush in induction curves of algae. A brief note on some of the results has been published in a congress report (Vejlby and Truelsen 1961).

3. Experiments

3 a. General experimental conditions

The study involved determinations of the carbon dioxide exchange of the plants, and in certain cases simultaneous determinations of carbon dioxide and oxygen exchanges, by means of the diaferometer method (the gas thermal conductivity method), in every detail as described by Vejlby (1958 a, 1959 a). The plant material was left in the dark in the diaferometer for a period of one hour prior to the beginning of the measurements. By repetitive experiments on the same plant material a minimum dark interval of 30 minutes was inserted. A light intensity of 15,000 lux was used, and the temperature of the air surrounding the plant material was 15°C in all experiments. The rate of the air stream was 3 litres/hour. The air was used in a state of complete water saturation.

The present study comprises experiments with clones of *Chlorella pyrenoidosa* under different conditions of cultivation and different measurement conditions. Poisoning experiments were carried out in this species by addition of iodoacetamide, and in other experiments the algae were subjected to intermittent illumination. The course of CO₂- and of O₂-time curves were determined for one of the strains. Determinations were further made of CO₂-time curves for a number of green, brown, and red algae from fresh water and from the sea. A Minor co-ordinated study comprises a few experiments with the moss species *Polytrichum attenuatum*.

3 b. Experiments with *Chlorella* grown at high concentrations of carbon dioxide

Measurements made in gas of high carbon dioxide content. The experiments were made with a clone of *Chlorella pyrenoidosa* Chick, placed at our

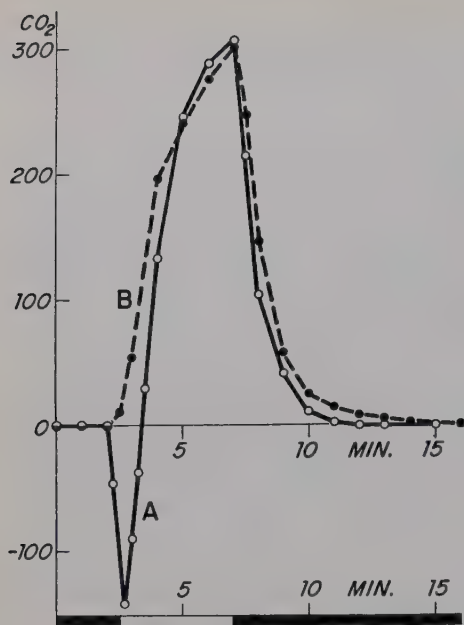


Figure 1. Photosynthesis-time curves (CO_2 -curves) for cells of *Chlorella pyrenoidosa* grown in a nutrient solution through which was bubbled 5 % CO_2 . Curve A measured in atmospheric air with the addition of 3 % CO_2 . Curve B measured in atmospheric air (0.030 % CO_2). Abscissa, time in minutes. Ordinate, uptake of CO_2 in arbitrary units (mm galvanometer readings). White and black areas under the axis of abscissas indicate light and dark periods, respectively.

disposal by the Institute for Spore Plants at the University of København. The algae were grown in 250 ml flasks in a nutrient solution described by Benson *et al.* (1949). Carbon dioxide enriched atmospheric air (5 % CO_2) from a bomb was bubbled through the nutrient solution. The flasks were placed in diffuse daylight on a glass covered veranda and were given additional illumination during the night (fluorescent tubes).

The algae cultures were centrifuged onto filter paper which while moist was placed in the plant chamber of the diaferometer. No attempt was made to measure the uptake or output of carbon dioxide quantitatively; hence the quantities of plant material were chosen merely large enough to give a suitable response on the diaferometer galvanometers when the algal cells were illuminated.

Figure 1 shows an example of a CO_2 -time curve measured with plant material from cultures such as the above (curve A); the measurements were made in atmospheric air to which had been added 3 % of carbon dioxide. The light gush is distinct and strong and lasts a good minute. An uptake of carbon dioxide then sets in, increasing steadily until the maximum photosynthetic rate under the given conditions is reached. (The experiment represented in Figure 1 by curve A was interrupted before steady state photosynthesis had been obtained). In no case was a primary peak or a tendency to the formation of one observed in these experiments. When illumination is discon-

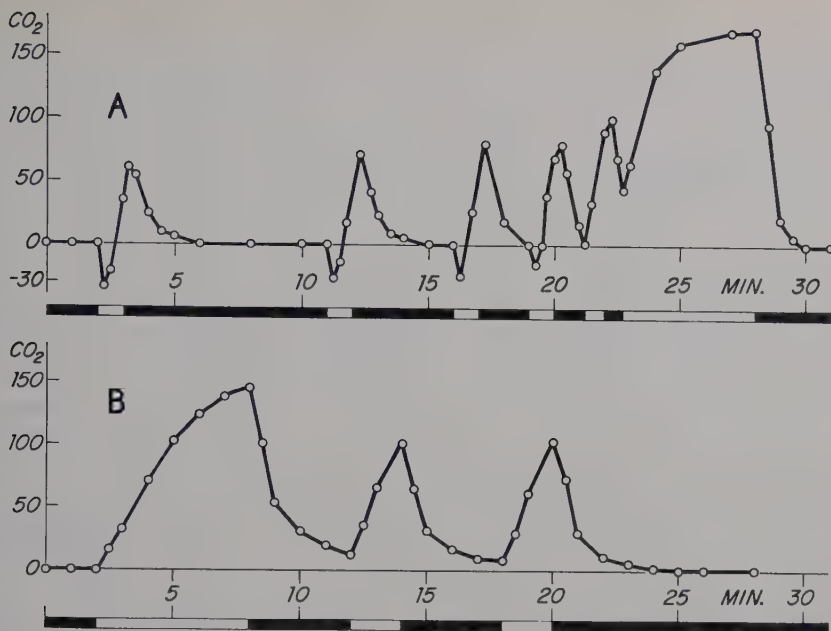


Figure 2. Photosynthesis-time curves (CO_2 -curves) for cells of *Chlorella pyrenoidosa* determined in atmospheric air with the addition of 3 % CO_2 . Curve A, cells grown in nutrient solution through which was bubbled 5 % CO_2 . Light periods of 1 minute interrupted by dark periods of 8, 4 and 2 minutes and of 75, and 45 seconds, respectively. Curve B based on experiments with plants grown in liquid medium through which atmospheric air (0.030 % CO_2). was bubbled. Light periods of 6, 2, and 2 minutes interrupted by dark intervals of 4 minutes. Otherwise as Figure 1.

tinued the curve decreases evenly down to the axis of abscissas (the respiratory level). In no case was it possible to observe an uptake of carbon dioxide after the onset of the dark period.

The formation of the light gush is in complete agreement with previous observations made by Emerson and Lewis 1941. However, in one respect do our CO_2 -time curves differ from these authors' results. In no case were we able to demonstrate a vigorous uptake of carbon dioxide immediately the light source was removed (a dark peak in the time curve) as is shown in Figures 3 and 4 of Emerson and Lewis.

Measurements made in atmospheric air. Measurements were then made in pure atmospheric air (0.030 % CO_2) with algae from the same culture which was giving light-gush curves in atmospheric air containing 3 % CO_2 . Curve B, Figure 1 shows one of several identical results. No light gush at all occurs, nor any primary peak, the curve being a typical half-arch one.

Measurements made in intermittent light. Curve A, Figure 2 shows a CO₂-time curve under conditions of alternating light and dark measured in atmospheric air with additional carbon dioxide (3 % CO₂). The algae cells originate from cultures through which air containing 5 % CO₂ was bubbled. One minute light periods (the approximate duration of the normal light gush) were interrupted by dark periods of 8, 4, and 2 minutes and of 75 and 45 seconds. The observation made by Emerson and Lewis, that the size of the light gush depends to a very great extent on the duration of the preceding dark period, is in complete agreement with the results obtained here. Dark periods of 75 and 45 seconds are not sufficiently long to allow the restoration of the CO₂ reservoir necessary for the formation of the light gush.

3 c. *Experiments with a moss species grown temporarily at high concentration of carbon dioxide*

Polytrichum attenuatum Menz. was placed for a period of one week in streaming atmospheric air to which 5 % of CO₂ had added under the same conditions of illumination used for the cultivation of the *Chlorella* material. The induction curve (measured in atmospheric air containing 3 % CO₂) showed no tendency to a formation of a light gush, but a normal primary-peak curve was found in all experiments, as is the case with moss plants of this species when grown under ordinary atmospheric conditions.

Hence cultivation at abnormally high concentrations of carbon dioxide does not appear to be sufficient to induce a light gush in all plants. However, it should be emphasized that only a minority of the cells affecting the measurements in the moss experiments had developed during the period spent in the air rich in carbon dioxide. Hence the results cannot without reservation be compared to the *Chlorella* experiments, where all of the cells used for the experiments had been under the influence of high concentrations of carbon dioxide during their entire period of formation and growth.

3 d. *Experiments with Chlorella grown at low concentrations of carbon dioxide*

Chlorella cells of the strain described in 3 b (the København clone) were grown in nutrient solution through which ordinary air was bubbled. Other conditions of cultivation were as above.

The results (exemplified in Figure 2, curve B, measurements in 3 % CO₂) show that in no case is a light gush obtained with plant material grown under low-carbon dioxide conditions. To complete the picture other experiments were made in which cultivation as well as the measurements of the

CO₂-time curves took place in pure atmospheric air. No light gush was found in this case either.

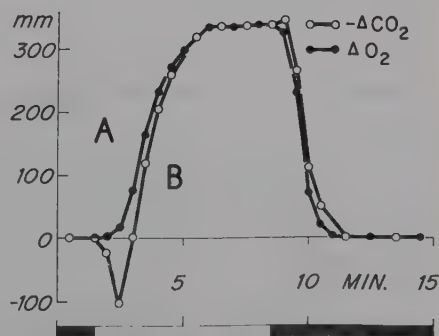
No formation of primary peaks was observed in any of the experiments included in groups 3 *b* and *d*. In all of the cases in which the experiments were extended to sufficiently long light periods and the determinations were made in ordinary atmospheric air only induction curves of the half-arch type were formed.

Hence it is established that the formation of a light gush in *Chlorella* is closely related to cultivation in a medium rich in carbon dioxide. The result is in perfect agreement with the facts which may be derived from the studies of McAlister and Myers' and of Gaffron's (see item 2, p. 578). It is further established that inability to form the light gush induced by the conditions of cultivation is not counteracted by the use of gas rich in carbon dioxide during the measurements, nor does an inherent ability to form a light gush develop when the measurements are carried out in atmospheric air poor in carbon dioxide.

3 e. CO₂-time and O₂-time curves for *Chlorella pyrenoidosa*

Simultaneous determinations of the oxygen and the carbon dioxide exchanged throughout the induction phase were made with the *Chlorella* strain (the København clone) used for experiments 3 *b* and *d*. The O₂-time and the CO₂-time curves (Figure 3, A, B) were calibrated against each other (it proved impossible to make absolute measurements in spite of several attempts) by assuming the quotient $\Delta\text{O}_2/\Delta\text{CO}_2$ to be 1.0 by the time photosynthesis reaches the steady state level. This assumption appears reasonable in view of available data (Vejlby 1959 a). The algae were grown in air containing 5 % of carbon dioxide, and the measurements for the curves were made in a gas mixture containing 3 % CO₂.

Figure 3. Photosynthesis-time curves for cells of *Chlorella pyrenoidosa* grown in nutrient solution through which was bubbled atmospheric air with the addition of 5 % CO₂; measurements in helium + 20 % O₂ + 3 % CO₂. A, oxygen-time and B, carbon dioxide-time curve. Units as in Figure 1, except that the ordinate represents, respectively, output of oxygen (curve A) and uptake of carbon dioxide (curve B).



Once more it is established that the light gush is not correlated to any fluctuations of the oxygen curve. Hence the results obtained by the diaferometer method are in complete agreement with those obtained by Emerson and Lewis (1941, two-vessel Warburg technique) and by Brown and Whittingham (1955, mass spectrometer investigations).

3f. The effects of iodoacetamide on the course of the light-gush curve

Massini (1957) found that iodoacetamide did not not significantly affect the reactions responsible for the appearance of the primary peak, but that the addition of this substance did very considerably reduce the steady state rate of photosynthesis. Leaves of *Dahlia*, *Datura*, and *Lycopersicum* were studied. Vejlby 1959 c found similar facts for the moss species *Polytrichum attenuatum*. Iodoacetamide was also used in *Chlorella* studies (Hiller and Whittingham 1959) in which it was found to completely inhibit the photosynthetic processes proper at a concentration of 10^{-4} M; but the size of the light gush was not significantly reduced by this treatment.

For our own experiments *Chlorella pyrenoidosa* (the København clone) were grown in 5 % CO_2 and the determinations were made in air containing 3 % of this gas. Prior to the measurements and to centrifugation the material was placed in a 10^{-3} M iodoacetamide solution for a period of 1 hour.

An example of the results is shown in Figure 4. The light gush appears to be unimpeded, but the induction curve proper rises only very slightly above the registered level for output of respiratory carbon dioxide.

Immediately upon discontinuation of illumination a peak may be observed in the curve, showing a sudden uptake of CO_2 in the dark. This uptake of carbon dioxide immediately the plant material is placed in the dark has previously been observed by Emerson and Lewis when they examined the formation of a light gush in *Chlorella* (1941, Figure 3). However, the curve peaks depicted there are not as pronounced as shown in our illustration.

The considerable uptake of carbon dioxide (the dark gulp) observed immediately after darkness sets in may possibly be due to the iodoacetamide

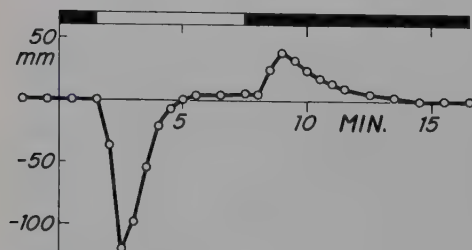


Figure 4. Photosynthesis-time curve (CO_2 -curve) for cells of *Chlorella pyrenoidosa* grown in nutrient solution through which atmospheric air with 5 % CO_2 was bubbled; measurements in atmospheric air with 3 % CO_2 . Prior to the measurements the *Chlorella* cells were treated with iodoacetamide for 1 hour. Otherwise as Figure 1.

treatment. In this connection it may be noted that in their 1959 studies Hiller and Whittingham were also able to register a vigorous uptake of Carbon dioxide immediately upon removal of the light source in cases where the *Chlorella* cells were affected by iodoacetamide. We have never been able to demonstrate a dark gulp in untreated material (see *e.g.*, curve A, Figure 1 and curve A, Figure 2). Probably this is due to the fact that the uptake of carbon dioxide (the gulp) is concealed when during the concluding phase of the induction period, immediately prior to the termination of illumination, the rate of uptake of carbon dioxide is very high and when during the first few minutes in the dark the time curve only slowly (probably only partly due to recording lag) descends to the level for output of respiratory carbon dioxide.

In Figure 3 (curve B) there appears to be a suspicion of an uptake of carbon dioxide during the first few minutes in the dark (*cf.* the courses of the oxygen and the carbon dioxide-time curves) in experiments with untreated material. In this case the conditions (measurements made in 77 % helium + 20 % oxygen + 3 % CO₂) allow a greater accuracy in the registration of changes in the carbon dioxide curve than when atmospheric air containing 3 % CO₂ was used, as was the case in the experiments on which Figures 1 and 2 are based.

3 g. *Bursty and non-bursty strains in Chlorella pyrenoidosa?*

As mentioned previously five clones of *Chlorella pyrenoidosa* were included in the studies of Hiller and Whittingham (1959). Two of these (Emerson's clone 3 and the Ellipsoidea-clone of Tamiya's) were found to give a light gush under suitable conditions; the remaining three did not. Hiller and Whittingham kindly provided us with a bursty and a non-bursty strain from their laboratory for comparison with our sample material.

The culture grown from the former clone (the bursty strain) was found to react in the same way as did the København clone, *i.e.*, it showed a light gush when cultivation and the determination of the induction curve took place in air rich in carbon dioxide, but not when cultivation or measurements took place in ordinary atmospheric air. The culture grown from the non-bursty strain at a high concentration of carbon dioxide (5 %) showed no light gush formation in agreement with the results of Hiller and Whittingham. However, in all of our experiments with this clone a peak formation (apparently not observed by Hiller and Whittingham) took place after about 1 minute, characterizing the induction curve as a typical primary-peak curve (Figure 5).

For a considerable period of time we considered this to be an example

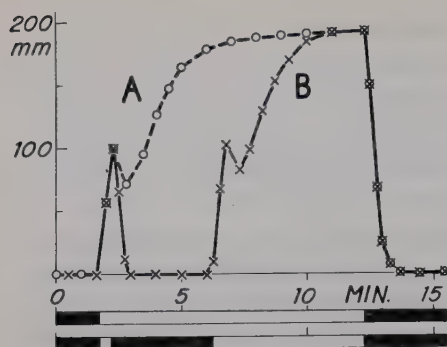


Figure 5. Photosynthesis-time curves (CO_2 -curves) for cells of *Oocystis* ("Chlorella pyrenoidosa, non-bursty strain") grown in nutrient solution through which was bubbled atmospheric air with 3 % CO_2 . Curve A, constant illumination for 10.5 minutes. Curve B, 0.5 minutes light, 4 minutes dark, 6 minutes light. Units as in Figure 1.

of the occurrence of primary-peak curves in *Chlorella pyrenoidosa*. As mentioned in section 2 this has previously been observed only by McAlister and Myers (1941). However, we had to revise this view following a detailed examination of the plant material (the non-bursty strain) by our taxonomic adviser Tyge Christensen. The examination showed "the algae in question to belong to the genus *Oocystis*, consisting with the description of *O. borgei* Snow, except that the cells are considerably smaller than otherwise stated for this species". Hence our results do not in any way support the contention that light gush curves and primary peak curves may be found together in the same species. The light gush curves were found only in experiments with *Chlorella pyrenoidosa* strains, and the primary peak curves only in experiments with the *Oocystis* strain. Hence physiologically speaking, from an induction curve point of view, the latter species is analogous to the unicellular green alga species of *Stichococcus bacillaris* (primary-peak curve observed by Harder and Aufdemgarten 1938 and Aufdemgarten 1939 a, b) and to a filiform alga belonging to the green alga genus of *Ulotrix* (primary-peak curve observed by van der Veen 1950).

The only remaining instance of an occurrence of primary-peak curves in *Chlorella pyrenoidosa*, then, are the 1941 results of McAlister and Myers', and the question arises if, as in our case, the "Chlorella" strain used for these experiments were not another organism altogether, mistakenly considered to be a strain of *Chlorella*? It is hardly possible even to solve this problem with certainty, but an approximation may be obtained by renewed simultaneous physiological and taxonomic-botanical studies of various strains of *Chlorella*. This type of study should also either invalidate or confirm the doubt raised by our results as to the occurrence of bursty and non-bursty strains in *Chlorella pyrenoidosa*.

It remains to draw attention to a peculiarity in the primary-peak curve for *Oocystis*. Usually in the case of mosses the removal of the light source

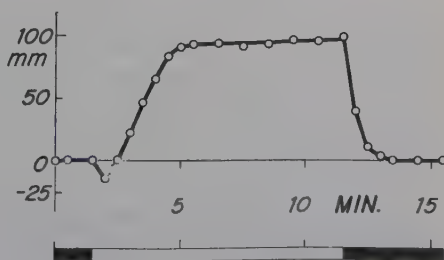
at the point where the primary peak is just fully developed results in an evolution of carbon dioxide, manifesting itself by the carbon dioxide-time curve descending briefly below the level of the respiratory output of carbon dioxide (Vejlby 1958 b, experiments with *Polytrichum attenuatum*). Under similar conditions this type of evolution of carbon dioxide is never found in *Oocystis*, the time curve does not descend below the level of respiratory carbon dioxide, but stays at this level until illumination sets in again. An example is seen in Figure 5, curve B. We have made no attempt to decide whether this phenomenon is specific for *Oocystis* cells or whether it occurs also in other primary-peak curves of green algae. We are unable to offer an explanation at the moment but hope to return to the subject after adding poisoning experiments and additional studies in intermittent light to our current data.

3 h. Additional studies in green algae

The occurrence of primary-peak curves in certain species (*Oocystis*, *Stichococcus*, and *Ulotrix*) induced us to investigate whether the primary-peak curve had a more wide-spread distribution throughout the system of green algae. Surprisingly, a filiform green alga from a fresh water aquarium through which atmospheric air is bubbled constantly, was found to show a slight, but fully reproducible, light gush (Figure 6). The alga in question was *Cladophora fracta* (Müll.) Kütz. This was our first example of an occurrence of light gush curves in plant material which had not been grown in a medium rich in carbon dioxide. As in all of the following experiments the measurements in this case were made in air containing 3 % CO_2 .

Further studies involved *Vaucheria dichotoma* (L.) Ag. and *Hydrodictyon reticulatum* (L.) Lagerheim, the experiments being made with algal cultures provided by The Institute for Spore Plants (courtesy of Tyge Christensen, M.Sc.). These species also produced a light gush without the benefit of growth in a medium rich in carbon dioxide. The gush was most pronounced in the case of *Vaucheria*, less certain in that of *Hydrodictyon*.

Figure 6. Photosynthesis-time curve (CO_2 -curve) for *Cladophora fracta* grown in an aquarium through which was bubbled atmospheric air; measurements made in atmospheric air to which 3 % CO_2 had been added. Otherwise as Figure 1.



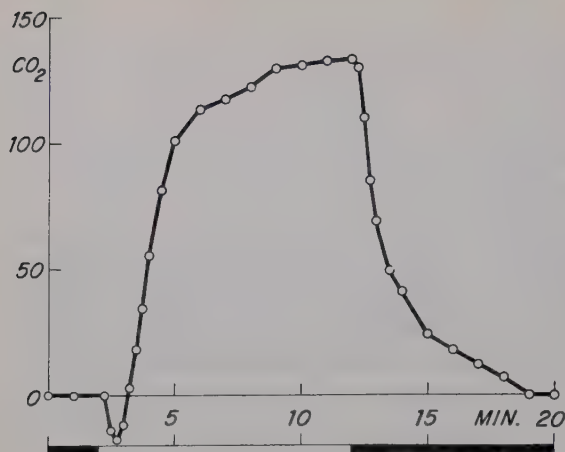


Figure 7. Photosynthesis-time curve (CO_2 -curve) for the brown alga *Ascophyllum nodosum*. Brought directly from its natural habitat, measurements made in atmospheric air with 3 % CO_2 . Units as in Figure 1.

We also had the opportunity to study one marine species, i.e., *Cladophora rupestris* (L.) Kütz.; measurements being made immediately it was brought in from its natural habitat, this species also gave distinct light gushes.

3 i. Experiments with brown algae

Brown algae were collected near the beach or were provided by the Marine Biology Station at Helsingør (Elsinore) whose material in all cases originates from the natural habitats of the plants (courtesy of Dr. G. Thorson, the director of the institute). A total of 5 species were studied, all measurements being made in air containing 3 % CO_2 .

Fucus serratus L., *Fucus vesiculosus* L., and *Ascophyllum nodosum* (L.) Le Jol. gave induction curves with light gush. An example is shown in Figure 7. A primary-peak curve was found for *Desmarestia aculeata* (L.) Lamouroux, while the curve for *Chorda filum* (L.) Stackh. was a pure half-arch. The results proved to be reproducible.

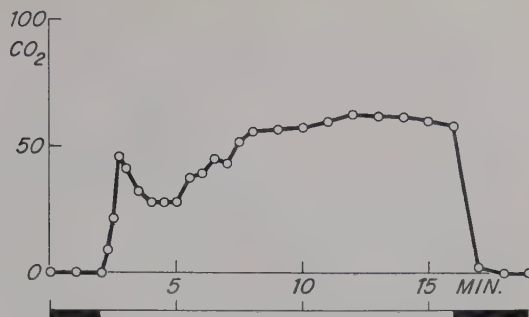
3 j. Experiments with red algae

Sample material was collected as above. Eight species were included in the examinations, all measurements being made in air containing 3 % CO_2 .

In no case was a light-gush curve found. Half-arch curves were found for three species, i.e., *Ceramium rubrum* Huds., *Polysiphonia elongata* (Huds.) Harv., and *Rhodomela confervoides* (Huds.) Silva.

Primary-peak curve were observed with reproducible certainty in four cases, i.e., *Phycodrys rubens* (Huds.) Batt., *Phyllophora membranifolia*

Figure 8. Photosynthesis-time curve (CO_2 -curve) for the red alga *Deleseria sanguinea*. Brought directly from its natural habitat, measurements made in atmospheric air with 3 % CO_2 . Units as in Figure 1.



(Good. et Woodw.) J. Ag., *Furcellaria fastigiata* (L.) Lamouroux, and *Deleseria sanguinea* (Huds.) Lamouroux (Figure 8). Probably a primary peak occurs on the induction curve for *Membranoptera alata* (Huds.) Stackh. as well; our material is too slight to establish this fact with sufficient certainty.

Discussion

Table 1 presents a review of our current knowledge of the incidence of the three types of induction curves within the various groups of the taxonomic system. With respect to the primary-peak curves of lichens, mosses, and higher plants the data included in the review is based on a list given by Vejlbj 1961, where details concerning species and references may be found.

Obviously it is impossible to treat the material presented in Table 1 statistically with any particular degree of accuracy, the observations being as yet far too few and too scattered within the various taxonomic groups. However, a distinct tendency is found for the primary-peak curve to be the exclusive induction curve type for all cormophytes from the mosses and upwards through the system. The primary-peak curve appears to be prevalent in the case of the red algae as well, whereas in brown and green algae both the primary-peak curve and the light-gush curve are found, albeit with a distinct tendency towards dominance on the part of the latter one. A primary-peak curve was found only in one out of 5 brown species studied. Primary-peak curves were observed with certainty only in three out of the 10 species of green algae studied. In addition, we have the algal species (probably belonging to the genus *Trebouxia*) which may be considered to be responsible for the formation of primary peaks in the induction curves of the lichen species examined.

The half-arch curve, *i.e.*, the pure induction curve devoid of fluctuations, has been found in all of the taxonomic groups studied, but under normal

Table 1. Survey of the reactions of different plant species on transfer from darkness to light as shown by the photosynthesis induction curve for CO₂ uptake. 15–20°C.

Plant groups and species	Curve with primary peak	Curve with light burst	Half-arch curve	References
Monocotyledons, 5 species	+			Aufdemgarten 1939 a
Dicotyledons, 19 species			+	
Coleus sp.				
Gymnospermae, 2 species	+			
Pteridophyta, 2 species	+			
Bryophyta, 17 species	+			
Helodium blandowii			+	
Lichenes, 2 species	+			
Rhodophyceae				
Phycodrys rubens	+			
Phyllophora membranifolia .	+			
Furcellaria fastigiata	+			
Delesseria sanguinea	+			
Membranoptera alata	(+)			
Ceramium rubra			+	
Polysiphonia elongata			+	
Rhodomela confervoides			+	
Phaeophyceae				
Desmarestia aculeata	+			
Fucus serratus		+		
Fucus vesiculosus		+		Emerson and Lewis 1941 McAlister and Myers 1941 van der Veen 1950 Brown and Whittingham 1955 Massini 1957
Ascophyllum nodosum		+		
Chorda filum			+	
Chlorophyceae				
Chlorella pyrenoidosa		+		
Chlorella pyrenoidosa ?	+	+		
Protococcus olivaceus		+		
Scenedesmus obliquus		+		
Scenedesmus sp.			+	
Vaucheria dichotoma		+		Harder and Aufdemgarten 1938 van der Veen 1950
Cladophora rupestris		+		
Cladophora fracta		+		
Hydrodictyon reticulatum ..		(+)		
Stichococcus bacillaris	+			
Ulotrix sp.	+			
Oocystis (borgei ?)	+			

conditions the incidence appears to be but slight. In the case of green algae it may be induced by specific conditions. When *Chlorella* is grown and tested at high concentrations of carbon dioxide (5 to 3 %) light gushes appear, but they are absent when growth and measurements take place in atmospheric air, in which case a typical half-arch curve is found instead (sections 3 b and 3 d). The half-arch curve is also found when green algae which normally show formation of the primary peak are grown in a special

nutrient solution poor in nitrogen (*Stichococcus bacillaris*, Aufdemgarten 1939 b). Among the cormophytes a *Coleus* species was found to produce the half-arch curve as demonstrated by Aufdemgarten, 1939 a, but its occurrence could not be confirmed by later studies under conditions inducing the formation of primary peaks in other dicotyledons (Vejlby 1961). In the moss species *Helodium blandowii*, however, it is only possible to show the existence of half-arch curves (Vejlby 1959 b). It is possible experimentally to influence the formation of the half-arch curve in mosses (*Polytrichum attenuatum*, Vejlby 1958 a) by increasing the temperature from 13 to 30°C. The change in temperature causes the primary peak to disappear. After cooling the ability to form the primary peak is recovered.

It appears reasonable to assume that the course of the half-arch curve (its rate of uptake of carbon dioxide keeps pace with the output of oxygen, cf. section 3 e) represents the uptake of carbon dioxide in the ribulose diphosphate system. Bassham *et al.* 1956 showed the concentration of compounds in this acceptor system to increase immediately upon the onset of illumination and to decrease to a certain, not measurable value shortly after illumination is discontinued (*Scenedesmus* experiments). The primary peak represents another acceptor system (Vejlby 1959 c) differing from the ribulose system by, among other things, being only in a minor degree affected by iodoacetamide. The biochemistry of the primary peak system is not known, but it is characterized by an apparent need for a steady supply of a certain minimum quantity of light energy in order to keep the carbon dioxide in a fixed state (Vejlby 1961). Usually, if illumination is suspended during the formation of the primary peak a gush of carbon dioxide appears (dark gush). During longer periods of illumination (6 minutes and above) the carbon dioxide is transferred completely from the primary-peak system to the ribulose system (Vejlby 1959 c).

Theoretically, the particular phenomena responsible for the presence or absence of the primary peak may be explained in two ways. 1) A dark period of a certain length may be required for the activation of the acceptor system (minimum 45 seconds, Vejlby 1961). Short dark periods then will prevent the appearance of the primary peak due simply to a shortage of available acceptor when illumination sets in. 2) Another explanation may be that the capacity in the primary-peak system is not subjected to fluctuations during the light-dark shift, but that short dark periods prevent the ribulose system from reaching the concentration minimum. Hence, if illumination sets in the ribulose system dominates the primary-peak system causing absence of the primary peak. The latter explanation appears to be the more likely one, it provides a feasible explanation of the absence of the primary peak at higher temperatures, since the concentration acceleration of the ribulose system

may well increase with increasing temperatures. The fact that it has not proved possible to demonstrate the existence of the primary peak in all species within the individual taxonomic groups where it mainly occurs may be due to fluctuations in the acceleration capacity of the ribulose diphosphate system from species to species.

The primary-peak system of the *Oocystis* clone appears to differ from that of *Polytrichum* in that it is impossible to force it into the formation of a dark gush (section 3 g). However, it is necessary to study this particular phenomenon in greater detail before it can offer suitable material for a rewarding discussion. Additional investigations are also needed to finally settle the question of the occurrence of the primary-peak curve in the case of *Chlorella* (section 3 g).

The present paper offers a number of new observations concerning the formation of light gush in induction curves. *Chlorella pyrenoidosa* has been shown to form a light gush only when the cells are cultured in air of high carbon dioxide concentrations (3 to 5 %) and when the determinations are made in carbon dioxide enriched air as well. The formation of light gush occurs only in algae and it appears to be impossible to induce it in mosses by growing them in air containing 5 % CO₂; however, the experiments carried out are not sufficient to furnish unequivocal evidence (section 3 c). For algae other than *Chlorella*, green as well as brown, light gush curves were demonstrated in plant material grown in equilibrium with the carbon dioxide concentration of atmospheric air.

The light gush formation must be based on a carbon dioxide acceptor system different from the ribulose diphosphate system (it remains unimpeded by iodoacetamide) as well as from the primary-peak system (it reacts in the opposite way). As yet no attempt has been made to determine whether the light-gush system is closely related to the chloroplasts as has been demonstrated in the case of the primary-peak system by Vejlby (1960 c). The comparatively slow build-up of the light-gush system in the dark (section 3 b) might indicate that this is based only on an uptake of respiratory carbon dioxide. However, occasionally a certain amount of carbon dioxide appears to be taken up at relatively rapid rate, resulting in the formation of a peak on the CO₂-time curve immediately upon the discontinuation of illumination (*Chlorella*, Emerson 1941). Inhibition of the ribulose system by means of iodoacetamide promotes the dark peak formation (the dark gulp, section 3 f). This suggests the existence of a process of transfer of carbon dioxide from the ribulose system to the light gush system, in such a way that when the former system operates at its maximum rate immediately prior to the onset of darkness, a transfer prevents the formation of a dark peak in the CO₂-time curve.

If this theory is correct, the ribulose system represents an intermediate between the primary-peak system and the light-gush system. Carbon dioxide may be transferred from the primary-peak system to the ribulose system, and from the latter to the light-gush system. No closer connexion appears to exist between the primary-peak system and the light-gush system. A fair amount of evidence favours the view that the occurrence of primary-peak curves and light-gush curves are characteristics of the individual plant species.

Summary

A study has been made of the occurrence of the three types of induction curves (CO_2 -time curves), *i.e.*, the primary-peak curve, the light-gush curve, and the half-arch curve, in 8 species of red algae, 5 species of brown algae and 6 species of green algae.

The results are reviewed in Table 1.

The primary-peak type of curve dominates among the red algae as well as among the cormophytes. Both primary-peak curves and light-gush curves are found among the brown and the green algae; the light-gush type occurs most frequently. A small number of half-arch curves are found within all of the taxonomic groups studied.

In the case of *Chlorella* the light-gush curves appear only when the cells are grown in a medium enriched in carbon dioxide (air containing 3 to 5 % CO_2 is bubbled through the nutrient solution) and when the determinations of the carbon dioxide exchange are made in air rich in carbon dioxide. Other species of green algae show light-gush curves when grown in media in equilibrium with the carbon dioxide content of ordinary atmospheric air, as do some of the species of brown algae included in the present study.

The CO_2 -acceptor systems responsible for the formation of the various types of induction curves are discussed.

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Influence of Surface Illumination on Plankton Photosynthesis in Danish Waters (56° N) throughout the Year

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1. Introduction

Investigation during the last 10 years has shown that the annual net primary production in all the oceans is of the order of magnitude of $1.2\text{--}1.8 \times 10^{10}$ tons of carbon (Steemann Nielsen and Aabye Jensen 1957, Ryther 1960). Quoting Müller (1960), this means that on the average only about 0.1 per cent of the light energy ($0.7\text{--}0.3 \mu$) penetrating the sea surface is utilized, while according to the same author 0.24 per cent of the light energy ($0.7\text{--}0.3 \mu$) reaching the surface of land is fixed in the terrestrial plants. In special cases about 2–3 per cent of the light energy ($0.7\text{--}0.3 \mu$) is fixed (net production) in terrestrial vegetation. By growing the plankton alga *Chlorella* in pilot plants under natural light conditions but with a continuous supply of carbon dioxide, even higher rates of light utilization (12 per cent) have been found provided the radiation energy during the day was low (Tamiya 1957). Under special conditions rather high yields may also be obtained in Nature in very shallow localities. Under general conditions, however, only a very small part of the radiation penetrating the surface of the sea is utilized in photosynthesis.

Taking the average for the whole year, the light factor is not normally limiting for primary production in the sea. Generally, only a small part of the submarine light is absorbed by photosynthetically active pigments in the plankton-algae and transferred into chemically bound energy. Most of the light is absorbed by the water, by particles of inorganic and organic origin, and by organic substances dissolved in the water.

At higher latitudes during winter, light is ordinarily the most important limiting factor. However, during summer at higher latitudes, as in the Tropics, the different factors of importance for plankton production are matched in such a way that most of them seem to act simultaneously as limiting factors (Steemann Nielsen and Aabye Jensen 1957). Therefore variations from day to day in the light energy penetrating the surface of the sea are of importance for the rate of photosynthesis below a surface unit, even in periods of the year when light can hardly be considered as a factor limiting the primary production in general. This interesting problem, which has many rather complicated details, is the topic of the present paper. Only comparatively few investigators have dealt with this problem before (Steemann Nielsen and Aabye Jensen 1957, Bauer 1957).

2. General Remarks on the Dependence of Plankton Photosynthesis on Light

It must be made clear at once that in Nature the investigation of the dependence of plankton photosynthesis on light is an extremely intricate problem. An accurate systematic study would involve so many different single measurements and experiments that any practical treatment of the problem would be very difficult.

1) The light energy penetrating the surface of the sea varies according to the season, the time of the day, and the weather conditions. The variations involve both intensity and wave-length composition. When measuring light in relation to the light utilization of the human eye it is possible to use special units such as lux or foot candles, because the spectral sensitivity of the normal human eye can be considered always to be the same. Unfortunately, it is impossible to construct any units of "photosynthetic lux" of general applicability. The different plankton components in the sea have varying spectral sensitivities. This is also the reason why it is not possible, when treating the dependency of plankton photosynthesis on the light energy in Nature, to present light energy unequivocally in energetic terms such as calories per unit of time and area, or in quanta per time unit and area. Regardless of the type of units used it is necessary to make approximations. Many specialists in photosynthesis still use lux as a unit for light intensity although this unit may be considered particularly obscure. The most important reason for doing so is psychological. If, for example, energetic units are used instead, the results can easily be misinterpreted. On the other hand, it is easy to convert lux into an energetic unit, if a special light source is used (cf. p. 598).

2) The spectral composition of the light varies with depth, but not in the same way in the different regions of the sea. Whereas blue light is predominant in the lower part of the photic zone in the oligotrophic tropical parts of the oceans, green light is predominant in identical layers in many other waters. Finally, red light may be predominant in special cases (Dvihally 1960).

3) Plankton algae adapt themselves to the light intensities found at their habitat, but variations due to the composition of species are very likely also of importance (cf. p. 604). In order to compute the rate of photosynthesis below a surface unit at a certain time of the year, a knowledge of the curves showing the rate of photosynthesis as a function of light intensity for the plankton populations in question will be necessary. The use of a casual curve for general purposes should definitely be avoided. Several authors have used as a standard curve the one presented by Jenkins (1937), which gives the rate of photosynthesis in a diatom culture as a function of light energy. In the view of present knowledge (Steemann Nielsen and Hansen 1959, Ryther and Menzel 1959), such a procedure may easily produce quite misleading results. In the present investigation, therefore, the establishment of appropriate photosynthesis-light curves for the different depths and for the different seasons of the year was greatly emphasized.

4) It is by no means possible to use the average light intensity during a whole day as a basis for the computation of the daily rate of photosynthesis at a certain depth. It is absolutely necessary to make the calculations for short periods and integrate them. In the present work, the rate of photosynthesis has been calculated for each hour of the day.

5) After-effects of high rates of illumination on the subsequent photosynthesis at both high and low light intensities are often of considerable importance, especially in the tropics at the surface. Even at higher latitudes this influence is of considerable importance during summer. The duration of such after-effects seems to vary a good deal. In the present work we have used continuous daily measurements of the light in Copenhagen published by Romose in 1940. As unit for the illumination a somewhat special unit of light intensity, the "B.J.Lux", was used (cf. Gabrielsen, 1940 p. 28). In the laboratory experiments with phytoplankton, ordinary lux were used. This is of very little importance, however.

It is somewhat difficult to compare photosynthesis in experiments carried out using incandescent light with photosynthesis under the conditions found in the sea. We have to consider both the difference in the wave-length composition of the light sources and the dependency of the photosynthesis on the wave-length composition.

Dvihally (1966) has presented a table according to which it is possible to

convert radiant energy of monochromatic light to lux. Such a conversion is of little use, however, if we want to consider the influence of light intensity on photosynthesis. As already mentioned, the spectral sensitivity of the plankton algae, although varying, has no correlation with the spectral sensitivity of the human eye which has its maximum in the green part of the spectrum. At present it seems more appropriate, as an approximation, to consider the light in the spectral region of $0.3\text{--}0.7\ \mu$ to be more or less uniform with regard to its influence on the rate of photosynthesis per unit of energy, and to consider $1,000\text{ Lux} = 1\text{ Klux}$ (incandescent light, $0.3\text{--}0.7\ \mu$) equivalent to $28.6 \times 10^{-2}\text{ cal/cm}^2 \times \text{h}$ (Gabrielsen 1948, p. 14) and 1 Klux (daylight $0.3\text{--}0.7\ \mu$), equivalent to $34.5 \times 10^{-2}\text{ cal/cm}^2 \times \text{h}$ (calculated according to Talling, 1957 p. 33). Daylight "B.J.Lux" have been put equal to ordinary daylight lux.

It is evident that a certain inaccuracy exists as a result of the heterogeneity of light sources and light measurements. However, this is not very important for the calculations made in the present paper. All available data suggest that the influence of the heterogeneity of light measurements is far less important than the influence due to variations in light intensity between bright days and average days.

3. Special Physiological Investigations of the Phytoplankton in the Sound

In contrast to most other seas, the Danish waters are characterized by a very strong vertical stratification of the water masses during all seasons. This is due to the fact that the Baltic water of low salinity runs out through the Danish waters and thus produces a light surface layer above a layer of more saline — and thus heavier — water from the North Sea running into the Baltic. Generally, however, the hydrographic conditions in the Danish waters cannot be described completely by only two definite water masses, one above and one below a sharp discontinuity layer, but better by a vertical series of lamellae of water.

Both Steemann Nielsen and Hansen (1959), and Ryther and Menzel (1959) have shown that the plankton population at the varying levels within the euphotic zone are adapted differently to light intensity if the zone includes different water masses, whereas no difference in adaptation is found if vertical mixing proceeds throughout the extension of the euphotic zone. It must therefore be strongly emphasized that not all of the results obtained during the present investigation can be directly used when discussing areas without a permanent stratification of the productive water masses. This is of particular importance when discussing the conditions during winter at higher

latitudes. If no stratification is found due to differences in salinity the water masses are vertically mixed during winter. No thermocline is found during that season.

Investigations were made in the Sound during a whole year, ordinarily once every month by MS "Ophelia" of the Marine Biological Laboratory in Helsingør (Elsinore). A station was made halfway between Helsingør and the Swedish coast. Besides making ordinary series of observations of temperature and salinity, the depth was determined at which 5 per cent green surface light was measured by means of a submarine photometer. In the area in question this depth corresponds approximately to the depth at which 2 per cent of all photosynthetically active light penetrating the surface is found. From this depth as from the surface, water was collected with a special non-metallic water bottle, transferred to thermosbottles, and immediately brought to the laboratory in København.

Here experiments were started at seven different light intensities (incl. dark). The temperature was adjusted to correspond to that found at the depth in question in Nature. The experiments lasted 3 hours. The experiments with surface water were made first, followed by those with the water from the depth having 5 per cent green light. The light intensity was varied by using filters placed in front of the bottles (cf. Steemann Nielsen and Hansen 1959 a). Duplicates were made at each light intensity including dark. The rate of dark fixation has been subtracted from all rates obtained at the different light intensities. Neutral filters from Schott and Genossen were employed. These filters meet all requirements. It must be noted that the light absorption is constant throughout the parts of the spectrum where photosynthesis takes place and where the photocell is sensitive to light.

A special arrangement was made in order to handle many bottles and different filters simultaneously; cf. Figure 1, showing the wheel with 12 bottles at the front and 2 dark bottles at the back. A special cover fitting 10 neutral filters may be placed in front of 10 of the 12 bottles at the front side of the wheel. This wheel is lowered into a water bath, where the temperature is constant to $\pm 0.2^{\circ}\text{C}$, although the water bath is illuminated by a row of nine 100 Watt incandescent bulbs. The maximum light intensity obtained is ordinarily 30,000 Lux.. By special arrangement it can be increased to 45,000 Lux.

The carbon-14 technique (Steemann Nielsen 1952) was used for measuring the rate of photosynthesis. The precision of the single measurements was ordinarily ± 8 per cent at all light intensities. Duplicates were always made. The precision was lower in some experiments where, *i.e.*, the concentration of plankton was very low. However, it was always possible to obtain sufficiently exact curves.

As the carbon-14 technique yields a value intermediate between net and gross photosynthesis if the duration of the experiments is short, it is necessary to make a correction in order to obtain values either for true gross photosynthesis or net photosynthesis (Steemann Nielsen 1958). This correction was always small. The rate of respiration varied in all cases between 5 and 10 per cent of the light saturated rate of photosynthesis. The rate of respiration was deduced by extrapolating to zero light intensity the curve showing the rate of net photosynthesis (corrected) as a function of light intensity; for details see Steemann Nielsen and Hansen (1959 a).

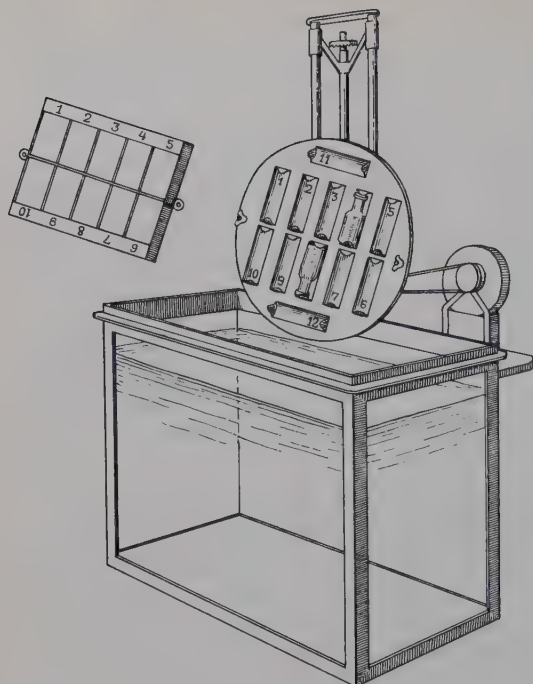
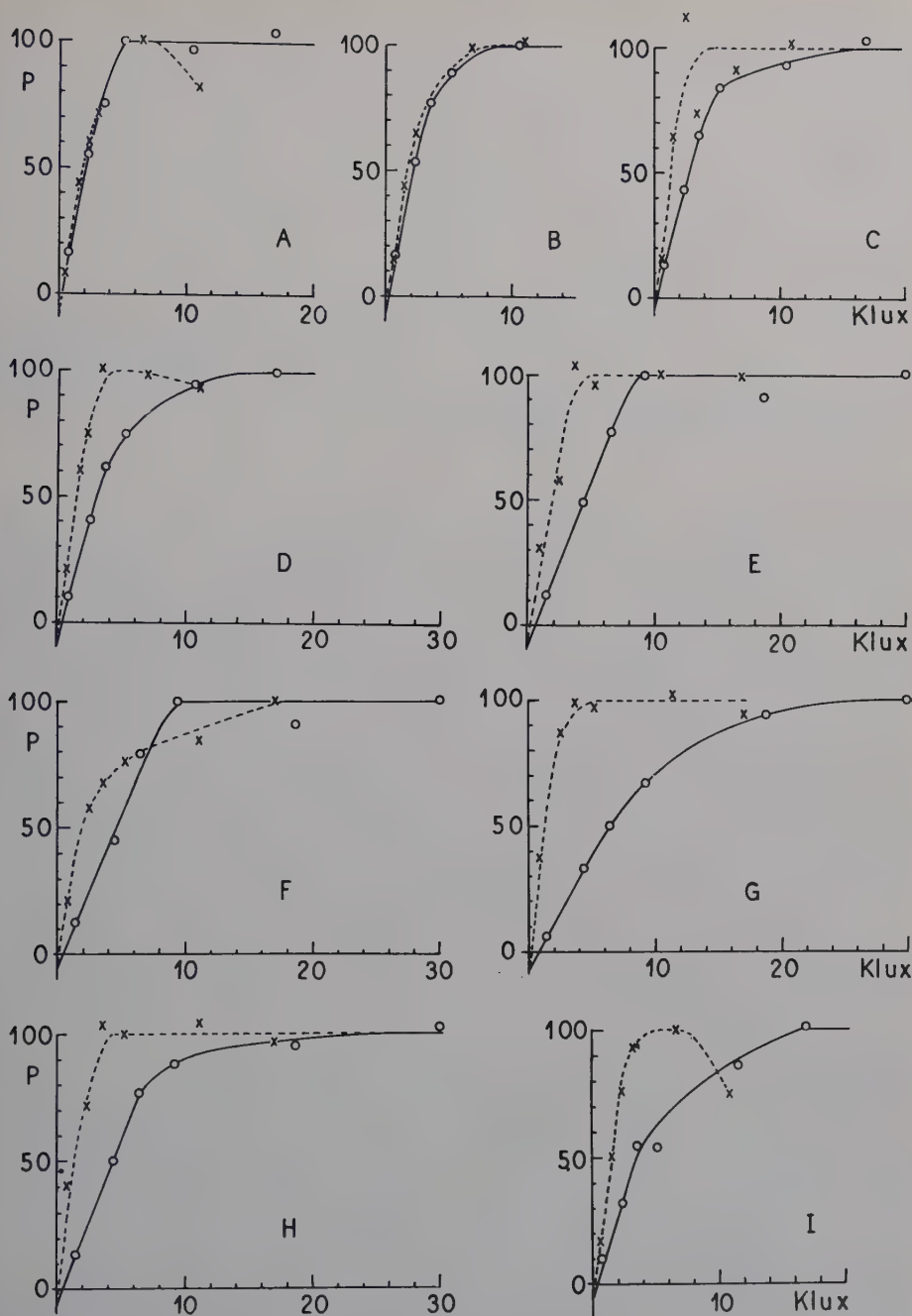


Figure 1. *The apparatus for measuring the rate of photosynthesis.* The wheel for 12 bottles at the front and 2 at the back is lowered into the water bath during the experiment and is rotated by means of the motor shown to the right. The cover fitting 10 neutral filters is shown to the left. The cover is divided into 10 single compartments by walls preventing light penetrating from one compartment to another. The details of the water bath are omitted.

The rate of photosynthesis was measured for a given volume of water; this is appropriate when dealing with primary production. In physiological experiments it is not very useful, however. Ordinarily in such experiments the photosynthetic rates are presented per dry weight or per volume of the algae. When using cultures of a single species of plankton algae these properties are easily measured. However, this is not true of natural plankton. Photosynthesis may be presented here per unit of chlorophyll, although the variation in the ratio chlorophyll/organic matter is considerable and the measurement of chlorophyll may be relatively inexact, as the content of the latter may be too low due to incomplete extraction of the pigment, or too high due to the presence of "dead chlorophyll" (Krey 1958). Instead of chemically determining the concentration of chlorophyll it is possible to make a physiological determination (Steemann Nielsen and Aabye Jensen 1957).

Figure 2 A-I. *Curves showing net photosynthesis (relative) as a function of light intensity.* Sound off Helsingør (Elsinore); ○, surface water, ×, water from the depth where 5 per cent of green surface light was measured. A: December 4th 1958, B: Febr. 13th 1959, C: March 25th 1959, D: Apr. 22nd 1959, E: May 14th 1959, F: June 4th 1959, G: July 15th 1959, H: Oct. 20th 1959, I: Novemb. 19th 1959. 1 Klux = 1,000 lux.



Legend p. 600.

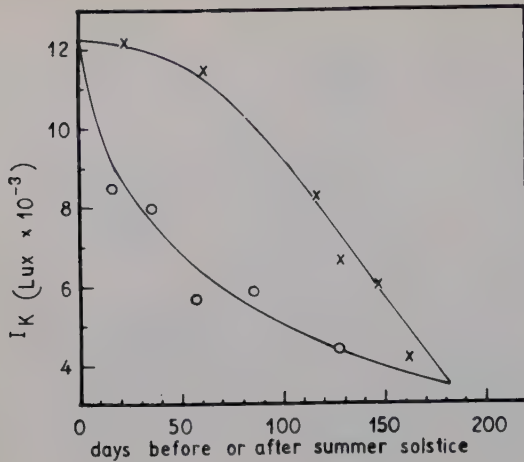


Figure 3. I_K (surface plankton) as a function of the number of days from summer solstice, ○: before, ×: after.

The rate of photosynthesis at low light intensities is proportional to the light absorption in the photosynthetically active pigments (cf. *e.g.*, Gabrielsen 1948). In the green alga *Chlorella vulgaris* grown at 30,000 Lux 0.29 mg C is assimilated per mg chlorophyll a+b per hour at 1,000 Lux (incandescent light, cf. Steemann Nielsen 1961). Although this ratio must vary when using algae as, *e.g.*, brown diatoms, it seems nevertheless appropriate as a first approximation to use the same ratio. We may call the unit "physiologically active pigment", admitting that other pigments may be involved in this unit.

In some few of the calculations to be presented in this article the rate of photosynthesis will be given per mg "photosynthetically active pigment". However, in Figure 2 A-I which present the different curves showing the rate of net photosynthesis as a function of light intensity, only relative rates are given, the rate at light saturation having in all cases been put at 100. Each figure includes both the curve for surface plankton and that for plankton from the 5 per cent light depth.

The curves for the plankton from the lower level are all very similar, all being typical of plankton from the lowest part of the euphotic zone ("shade" plankton, Steemann Nielsen and Hansen 1959). I_K varies only between 2,500 and 3,600 Lux and not according to the season. I_K is the light intensity at which the straight line representing the initial slope of the photosynthesis curve intersects the straight line representing the photosynthetic rate at light saturation (Talling 1957).

On the other hand, the shape of the curves for the surface plankton are extremely different, and vary according to the season. I_K varies between 4,200 and 12,100 Lux. This means that all kinds of surface curves are found,

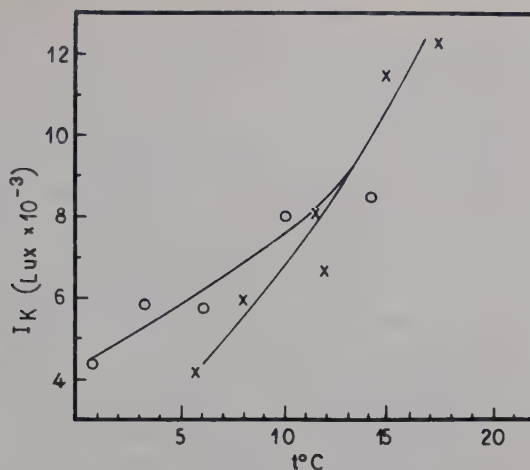


Figure 4. I_K (surface plankton) as a function of temperature, ○: before summer solstice, ×: after summer solstice.

varying from those typical of "shade" plankton (December-curve) to those typical of "sun" plankton (July-curve).

Figure 3 presents the I_K of the surface curves as a function of the number of days from summer solstice. A circle means before and a cross after. At the same time before and after summer solstice the light conditions at the surface may be considered to be essentially the same. The values of I_K fall on two definitely separate curves, one for those before and one for those after summer solstice. This indicates that the light adaptation of the algae is not due exclusively to the light conditions at the habitat of the algae. At least one other factor must be of importance for the shape of the curves showing photosynthesis as a function of light intensity.

This factor could be: a) a particularly long duration of the period involved in establishing a special light adaptation, b) the temperature, c) the composition of species. Experiments to be published elsewhere (Steemann Nielsen *et al.*, under preparation) have shown that plankton algae adapt fairly rapidly to a new light intensity. Therefore, the time factor is hardly of importance in this respect. Temperature, on the other hand, cannot be ignored. In Figure 4, I_K is presented as a function of temperature. Also here two definitely different curves are found, determined by the values for the time before or after summer solstice. The light conditions and the temperature vary by no means synchronously in the Danish waters, temperature being always delayed compared with light. When the day length is the same in spring and autumn, the temperature at the surface is much lower at the former season. For example on the two days for experiments in 1959, February 13, and October 31, the surface temperature was 1°C and 12°C,

respectively, although the days mentioned were 128 days before and after the summer solstice, respectively. This indicates that the adaptation to light of marine phytoplankton is a function both of the light intensity and of temperature. However, the situation is very likely still a little more complicated. The composition of species is undoubtedly also of importance. In Arctic Waters during summer Steemann Nielsen and Hansen (1959) found an I_K of about 9,000 Lux at about 5°C, corresponding to the conditions found in Danish waters in June when temperature was about 14°C, and the light conditions no worse, although the day is shorter than in the Arctic.

The different shapes of the light-photosynthesis curves of surface plankton during spring and autumn must be considered to be of some importance for the pattern of organic production, because the rate of respiration seems closely related to the rate of light saturated photosynthesis, such as, *i.a.*, shown on p. 599. In section 5 the question will be discussed. In fact, it is rather obvious that in Nature a rather close connection must be found between the rates of photosynthesis and respiration, each process making equally important contributions to growth.

4. Surface Illumination and the Site of the Compensation Depth

No continuous light measurements were made during the present investigations. However, as mentioned on p. 597, Romose (1940) has published such measurements made in København over a two year period. These measurements have been reconsidered. Figure 5 presents a) a curve based on the integrated average daily light for every month, b) a curve based on the average of the three darkest days during each month, and c) a curve based on the average of the three brightest days during each month. The curves are corrected for the fact that during spring the brightest days ordinarily will be found during the later part of the month and the darkest days during the first part (*vice versa* during the autumn).

Figure 5 demonstrates the considerable variations found throughout the year in the daily light energy penetrating the surface of the sea. On the average nearly 30 times more light energy reaches the surface in the middle of summer than in December. More than 100 times as much light penetrates the surface during a bright day in June than during a dark day in December.

On the other hand the variations between dark days and bright days at the same time of the year are relatively insignificant, cf. Figure 6. In the period April–September on the darkest days of the month the surface receives between 30 and 36 per cent of the light received on the brightest days. In the period November–February, the darkest time of the year, the percentage

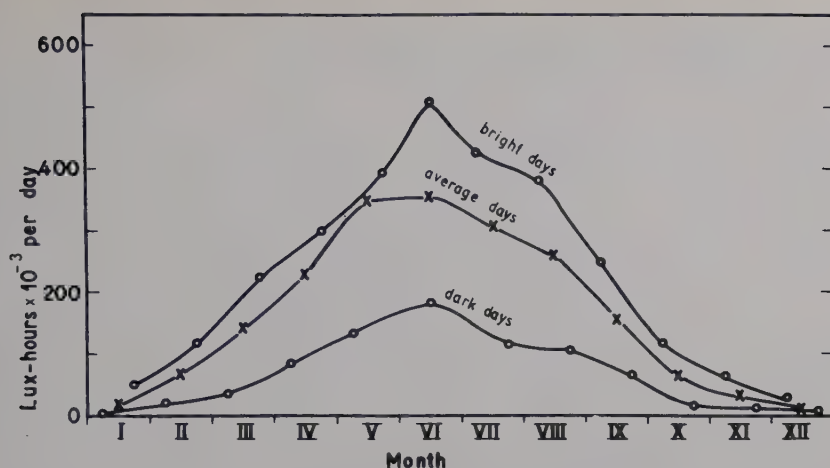


Figure 5. The variation of the integrated daily light throughout the year for bright, average, and dark days (cf. the text). Light measurements by Romose 1940.

is about 20, and finally for March and October about 26. The relatively low percentage in winter is very likely due primarily to the low altitude of the sun making the average path of the light rays through a cover of clouds much longer.

Steemann Nielsen (1954) showed for a tropical ocean under the condition of even distribution of phytoplankton within the photic zone, that even considerable variations in the illumination from day to day have only a limited influence on primary production below a surface unit. If the light falling

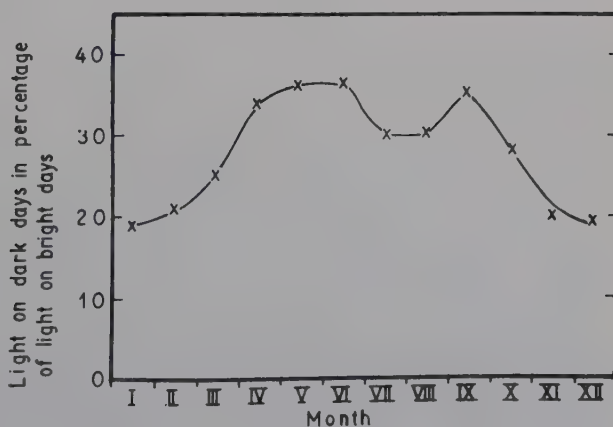


Figure 6. Light on dark days as a percentage of light on bright days throughout the year.

on the water surface is reduced throughout the day to one third, the rate of total photosynthesis is only reduced to two thirds.

In the Sound off Helsingør the production pattern is rather complicated. Due to the vertical stratification of the water masses several distinct laminae are found, each containing special plankton populations with often very varying capacities for the photosynthesis. If the main part of the phytoplankton is found in the uppermost part of the photic zone, light variations from day to day during summer have only a trivial influence on the total production below a surface unit. A moderate decrease in light may even increase the production. If on the other hand the main part of the phytoplankton is found in the lowest part of the photic zone, a decrease in light due to overcast weather will bring about a corresponding decrease in photosynthesis below a surface unit. The background for this striking difference in behaviour is the fact that whereas the rate of photosynthesis is proportional to light intensity at such low light intensities as are always found near the lower boundary of the photic zone, at real high light intensities — such as found near the surface during the middle of the day on bright summer days — a reduction of the photosynthetic rate takes place; for details cf., *e.g.*, Steemann Nielsen 1958.

The influence of variations in light on the total production below a surface unit would be rather complicated to show in the Sound. Hence we attempt in the following to demonstrate instead the influence of such variations, both from day to day and from season to season a) on the site of the compensation depth and b) on the production in the actual surface layer.

The measurements of the submarine light in the Sound off Helsingør during the present investigations and on other occasions have shown that the transparence of the water varies, but not very much, and not according to the season. The depth at which 5 per cent green surface light — equal to about 2 per cent of all photosynthetically active light — was found during the investigation of 1958–1959, is presented in Table 1. On the average one per cent of the sum of all photosynthetically active surface light is found at a depth of 20 m. The compensation depth — *i.e.* the depth at which the rates of photosynthesis and respiration compensate each other over a period of 24 hours — is therefore primarily dependent on the light penetrating the surface of the sea. As a simplification we have considered the transparency always to be the same — 1 per cent of all photosynthetically active surface light at a depth of 20 m.

For the plankton from the lower part of the photic zone, the curves showing the rate of photosynthesis as a function of light intensity were demonstrated in Figure 2 A–I. It seems reasonable to assume that the chlorophyll content in these “shade” algae is nearly the same throughout the year. The

Table 1. *The depths at which 5 per cent of green surface light was measured during the different excursions in the Sound off Helsingør. These depths correspond to the depths at which 2 per cent of all photosynthetically active surface light is found.*

Time	5 per cent depth
Dec. 12. 1958.	13
Febr. 13. 1959	22
March 25. 1959	17
Apr. 22. 1959.	17
May 14. 1959.	17
June 4. 1959.	14
July 15. 1959.	22
Oct. 20. 1959.	18
Nov. 19. 1959	18

relation total organic matter: chlorophyll is probably about 30, corresponding to the conditions in *Chlorella* grown at low light intensities; (cf. Kok 1953). Although all of the curves are very similar, nevertheless special curves obtained during the cruises in question have been used for the calculations presented below.

By means of Romose's light observations the average light intensity for every hour of the day was computed a) for an average day for each month, b) for the (three) brightest days of each month and c) for the (three) darkest days of each month. By means of these values and of the light photosynthesis curves we have determined the compensation depths for each month, a) for days receiving the average amount of light energy for the month, b) for the three brightest days and c) for the three darkest days. The respiratory rates were always put at 8 per cent of the photosynthetical rate at light saturation. This rate was the average of all measurements which, as mentioned above, varied but little inter se.

Figure 7 presents these values. The light intensity in percentage of the surface light ($0.3\text{--}0.7\ \mu$) is also shown. For days with average light conditions the compensation depth is found at a depth of about 18 m. (1.7 per cent of the surface light) during the middle of the summer and at 2 m. in December (60 per cent of the surface light). Considering only the three brightest days of each month, the depths are 19 m. and 5 m., respectively. Considering only the three darkest days of each month the depths are 15 m. and 0 m., respectively. This clearly shows that the day to day variations of the light conditions is of only slight importance for the depth of the photic zone during the middle of summer, but of decisive importance during the middle of winter when, during dark days, no organic net production can take place even at the actual surface. Figure 7 shows further that the depth of

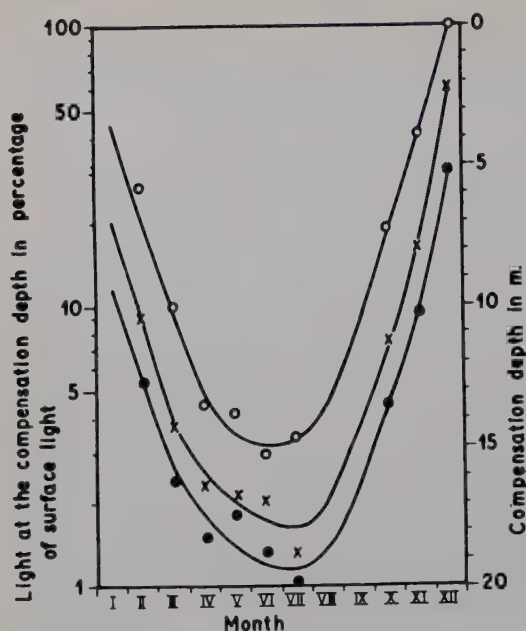


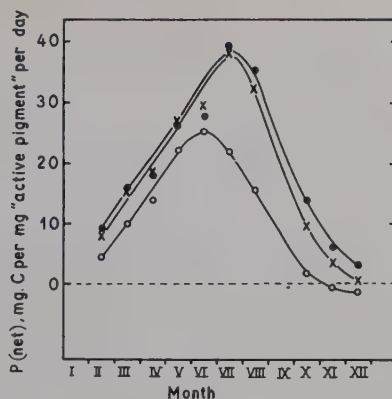
Figure 7. The variation of the compensation depth in m. throughout the year on bright days (●), average days (×) and dark days (○). Also shown are the light intensities (0.3–0.7 μ) at the compensation depth as a percentage of the intensity at the surface.

the photic layer is identical during spring and autumn if the number of days either before or after summer solstice is identical (and if the transparency of the water is the same).

5. Surface Illumination and the Production Rate in the Surface Layer

As mentioned in the Introduction the water masses off Helsingør are vertically strongly stabilized. According to daily measurements at Lappegrunden lightship (cf. Nautisk Meteorologisk Årbog, Yearbook for Nautical Meteorology, e.g. for 1959) the homogeneous surface layer rarely reaches down to a depth of 5 m. On the average only the 2 1/2 upper metres may be considered homogeneous. For this water layer the average rate of net photosynthesis — computed per mg. of “active pigment” (cf. p. 600) has been computed per 24 hours for all months of the year. In Figure 8 these rates are presented a) for an average day for each month, b) for the average of the three brightest days of each month, and c) for the average of the three darkest days of each month. The calculations were based on the curve for the month in question showing the rate of photosynthesis of surface plankton as a function of light intensity used. The rate of respiration was always put at 8 per

Figure 8. *The rate of net photosynthesis in the surface layer (0–2.5 m.) as a function of "active pigment" throughout the year. ●: bright days, ×: average days, ○: dark days.*



cent of the rate of light saturated photosynthesis (cf. p. 599). A standard transparency corresponding to that used in Figure 7 was employed.

As is to be expected, the variations throughout the year in the rate of net production are very considerable, varying for an average day of the month from close to zero in December to 37 mg. C per mg. active pigment in July. On average days during spring and autumn, when the number of days before or after summer solstice is the same, the rates of net production are virtually identical.

During spring and early summer this rate in the surface layer (0–2.5 m.) is only slightly higher on bright days than on average days. During late summer, autumn, and winter the differences are considerably more pronounced. Even in December a significant rate is found on bright days.

The rate of net production is always considerably lower on dark days than on days with average illumination for the time of the year. The differences, however, are most pronounced during the summer, autumn and winter (cf. Figure 8). As shown in Section 3 the algae at the surface are relatively better adapted to high light intensities after summer solstice than at a comparable time before solstice. Therefore the algae are a little better suited to utilize the high light intensities on bright days in the autumn than in the spring. However, as the rate of respiration is lower in the spring than in the autumn (cf. p. 604) the net production is superior on dark days in the spring as compared to corresponding days in the autumn. On average days the differences in light adaptation is of no great importance for the rate of net production.

Unfortunately, the rates of photosynthesis can be stated only per unit of "active pigment". It would have been better to present the rates per content of organic matter in the algae. It is extremely difficult to measure this con-

tent with any accuracy. No measurements of this kind have been made in the present investigations. However, available data show the ratio organic matter/chlorophyll in surface plankton to be considerably higher during midwinter than during midsummer, probably by a factor of two or even three (Steemann Nielsen and Hansen 1959). When using a factor of two, the rate of net production found per algal organic matter in the surface layer 0–2.5 m. on bright days in December is about 20 per cent of that during bright days in July, instead of the 10 per cent found when photosynthesis is computed per unit of pigment.

This explains why in years with relatively many bright days during December a considerable stock of plankton algae may survive in the surface layer throughout the month and give rise to a considerable rate of organic production during the bright days. Steemann Nielsen (paper under preparation) describes such conditions for the Kattegat. During the last seven years, measurements of primary production using the carbon-14 technique have been made every fortnight.

6. Discussion

The present study may be used for comparison with the investigations made during recent years on the rate of primary production in the Danish waters. Although samplings were made only from two depths and only once every month, it is possible by using the light measurements for average days to compute an approximate value for the annual rate of gross primary production per square meter of the surface. It is about 30 g. C.

During an investigation 1932–1933 (Steemann Nielsen 1958 b) an annual rate of gross production of 39 g. C/m² was found in the same area. The oxygen technique was employed at that time. The present investigation thus confirms the statement by Steemann Nielsen, 1958 b, that the Sound is a relatively unproductive area compared with other parts of the Danish waters, such as the Kattegat and the Great Belt. Several scattered investigations in the Sound made during recent years have given the same result.

The transparencies of the water masses in the Sound and in the Great Belt are very much alike. Although no systematic investigations have been made in the latter area, sufficient scattered observation on transparency are available.

Hence it appears permissible to compare the depths of the photic layer for the Sound off Helsingør and the Great Belt (Halsskov Rev) for the different months of the year. The values for average days obtained during the present investigation are used for the Sound. The in situ measurements made

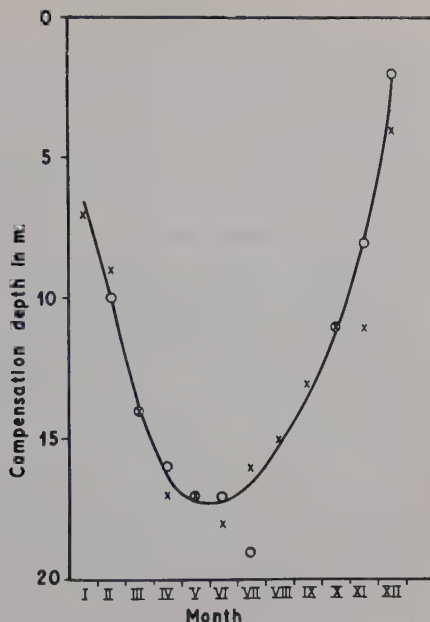


Figure 9. The compensation depth throughout the year in the Great Belt (x) and in the Sound (o); cf. the text.

every fortnight over a period of 5 years at Halsskov Rev lightship have been used to calculate the approximate depth of compensation for 24 hours in the Great Belt. This depth was defined as the depth at which the rate was 20 per cent of the maximum rate (February–October), for November and January 30 per cent and for December 50 per cent. Although this type of calculation can give approximate values only, Figure 9 shows a remarkable agreement with the values for the Sound obtained in quite another way.

7. Summary

Curves are presented showing the rate of photosynthesis as a function of light intensity for plankton collected in the Sound off Helsingør. The plankton was collected about once every month over a period of a year both from the surface and from the lower part of the photic zone.

The curves for the plankton from the lower level are all alike and typical of "shade" plankton. The shape of the surface curves are extremely different, varying from typical "shade" curves in winter to typical "sun" curves in summer. It is shown that the slopes of the curves are not determined solely by the light conditions but probably also to some extent by temperature and by the composition of species.

The site of the compensation depth throughout the year is determined both for bright and dark days and for days with average light conditions. In addition to daily light measurements the photosynthesis curves were used for computing the site of the compensation depth. The day to day variations caused by different light conditions are of decisive importance only during the dark time of the year. The variations between the seasons are considerable. It is shown that the compensation depth directly determined by "in situ" experiments is essentially identical with the depth determined by means of the above mentioned curves.

The production rate in the homogenous surface layer — about 2.5 m. deep — is determined throughout the year for bright days, average days, and dark days. The day to day variations caused by different light conditions are small during summer, but considerable during winter. The variation between summer and winter is high.

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Enzymes of the Electron Transport System in Rumex Virus Tumor Tissue

By

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Introduction

Numerous studies of the electron transport system in plants have been conducted (1) and it appears that the enzymatic reactions of this system are localized in a particulate (mitochondrial) cell fraction. As part of an investigation of the possible biochemical differences between normal and neoplastic plant tissues, this study deals with the electron transport system of *Rumex virus tumor tissue*. Previous investigations of the metabolism of this tissue have dealt with the respiration of tissue slices (2), oxidases present in cell-free extracts (3), and the capacity of a particulate fraction obtained from tumor cells to oxidize Krebs cycle intermediates (4). The present paper is concerned with the enzymes of the electron transport system in the tumor tissue as evidenced by the activities of a particulate fraction obtained from cells of this tissue.

Methods

The tissue was grown (2) and the particulate fraction was prepared (4) as previously described. All reagents were made up in buffer unless otherwise noted and the final volume of each reaction mixture was obtained by the addition of this buffer which consisted of KH_2PO_4 , 0.02 M; sucrose, 0.4 M and MgCl_2 , 0.005 M, at the indicated pH for each experiment.

Reduced diphosphopyridine nucleotide (DPNH), reduced triphosphopyridine nucleotide, (TPNH) and horse cytochrome c were obtained from the Sigma Chemical

Co. Antimycin A was obtained from the Wisconsin Alumni Research Foundation and dissolved in absolute alcohol for use; an equal volume of alcohol was added to control experiments. Cytochrome c was reduced by the addition of a small amount of sodium dithionite followed by aeration for ten minutes.

Experiments were conducted in 1-cm light path cuvettes in a Beckman Model DU spectrophotometer. Cytochrome oxidase was measured by the reoxidation of reduced cytochrome c. Cytochrome c reductases and cytochrome c reduction by succinate were assayed by the reduction of cytochrome c in the presence of KCN and DPNH or TPNH, or succinate, respectively. Oxidation of reduced cytochrome c and reduction of cytochrome c were measured at 550 m μ . Oxidation of TPNH and DPNH was measured by the change in optical density at 340 m μ ; diaphorase activity was measured at 600 m μ by the reduction of 2,6 dichlorophenolindophenol in the presence of KCN and DPNH or TPNH. Nitrogen was determined by the method of Thompson and Morrison (5); 0.1 ml. of the final particulate suspension contained approximately 25 μ g N.

Results

Experiments with tissue slices had previously revealed that the oxygen consumption of the tumor tissue was the same in an atmosphere of 100 % oxygen as in air, suggesting the presence of a cytochrome system (2). Cell-free extracts of the tissue have been shown to contain a cytochrome c oxidase

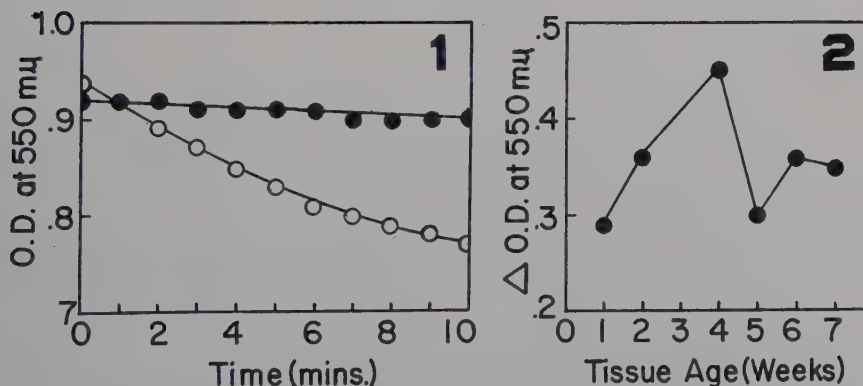


Figure 1. Cytochrome c oxidase activity of a particulate fraction from *Rumex virus tumor tissue*. Complete reaction mixture contained 2.0 mg. cytochrome c reduced with dithionite; 0.2 ml. particulate suspension; where indicated, KCN in a final concentration of 10^{-4} M; and sufficient buffer to make 3.0 ml; pH 7.3; temp. 26°C. Upper curve, plus KCN; lower curve, minus KCN.

Figure 2. The influence of age of tissue on cytochrome c oxidase activity. Complete reaction mixture as in Fig. 1. Activity indicated for 0.2 ml. of a particulate suspension containing 50 μ g N. Reaction time, 10 mins.

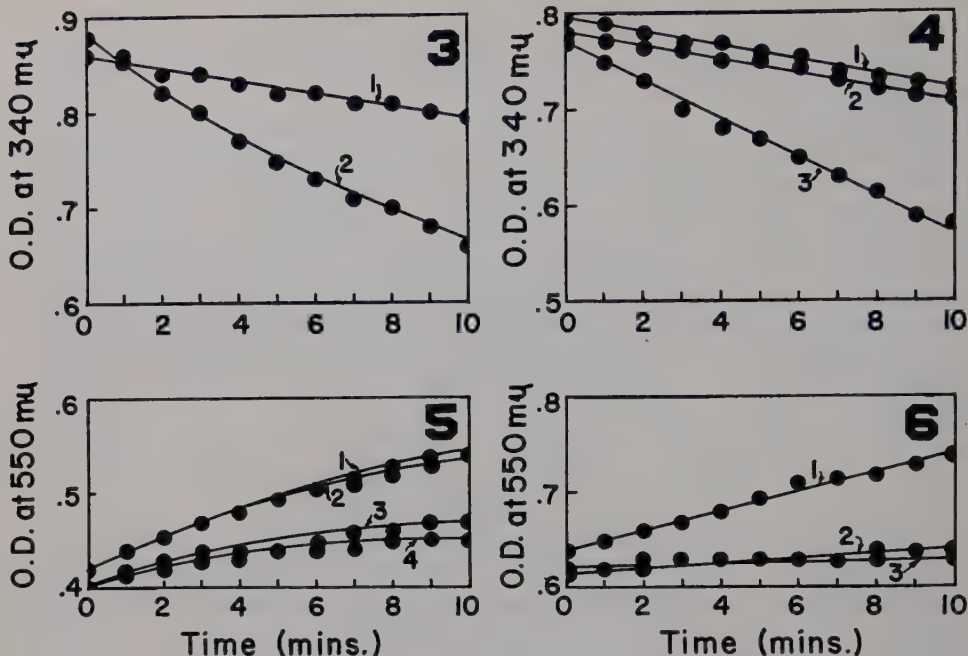


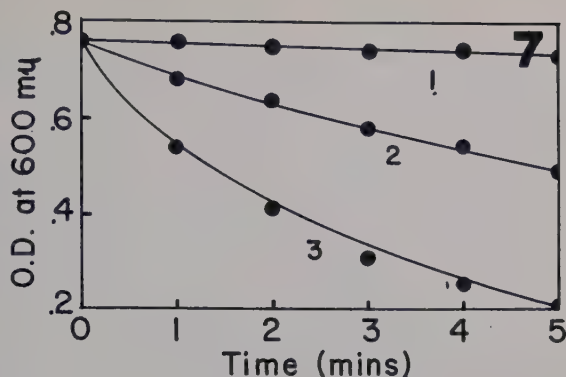
Figure 3. DPNH (Curve 2) and TPNH (Curve 1) oxidase activities of a particulate fraction from *Rumex virus tumor* tissue. Complete reaction mixture contained DPNH or TPNH, 5×10^{-5} M; particulate suspension, 0.2 ml.; and sufficient buffer to make 3.0 ml.; pH 7.0; temp. 26°C .

Figure 4. The effect of cyanide and Antimycin A on the DPNH oxidase activity of a particulate fraction from *Rumex virus tumor* tissue. Complete reaction mixture as in Figure 3; KCN, 10^{-3} M, or Antimycin A, $5 \mu\text{g}$ in 0.01 ml. added where indicated. Activity in absence of inhibitors shown in curve 3; in presence of Antimycin A, curve 2; in presence of KCN, curve 1.

Figure 5. The DPNH and TPNH cytochrome c-reductase activities of a particulate fraction from *Rumex virus tumor* tissue. Complete reaction mixture contained cytochrome c, 3×10^{-5} M; KCN, 10^{-3} M; DPNH or TPNH, 5×10^{-5} M; Antimycin A, where indicated, $5 \mu\text{g}$; particulate suspension, 0.1 ml.; buffer to make 3.0 ml.; pH 7.0; temp. 26°C . Curves 1 and 2 show DPNH-cytochrome c reductase activity in presence and absence of Antimycin A respectively; curves 3 and 4 show activity with TPNH in absence and presence of Antimycin A respectively.

Figure 6. The succinate-cytochrome c reductase activity of a particulate fraction from *Rumex virus tumor* tissue. Complete reaction mixture contained cytochrome c, 3×10^{-5} M; KCN, 10^{-3} M; succinate, 0.003 M; particulate suspension, 0.2 ml.; Antimycin A, where indicated, $5 \mu\text{g}$; final volume 3.0 ml.; pH 7.0; temp. 26°C . Curve 1, complete reaction mixture; curve 2, complete plus Antimycin A; curve 3, minus succinate.

Figure 7. The DPNH and TPNH diaphorase activities of a particulate fraction from *Rumex virus tumor* tissue. Complete reaction mixture contained DPNH or TPNH, 5×10^{-5} M; KCN, 10^{-3} M; 2,6 dichlorophenol-indophenol, 4×10^{-5} M; particulate suspension, 0.2 ml.; sufficient buffer to make 3.0 ml.; pH 7.0; temp. 26°C . Curve 1, minus DPNH and TPNH; curve 2, complete with TPNH; curve 3, complete with DPNH.



which was inactivated by heat and KCN (0.001 M). The cytochrome c oxidase activity of a particulate cell fraction and its inhibition by KCN is shown in Figure 1. Control mixtures containing a particulate fraction heated at 100°C for 10 mins. showed no measurable activity.

The particulate fractions used in the above experiments were obtained from tissues that were 7-weeks old. Since it has been shown that the rate of oxygen consumption may vary with the age of this tissue (6), the cytochrome oxidase activity of particles obtained from tissues from 1–7 weeks old was determined (Figure 2). Activity is based on the change in optical density at $550\text{ m}\mu$ in 10 mins. for fractions containing $50\text{ }\mu\text{g N}$ each. Although there is some variation in cytochrome oxidase activity among tissues of the same age, it appears that a maximum activity is reached when the tissue is about 4 weeks old; younger and older tissues exhibit lower activity.

The particulate fraction contained an active DPNH and TPNH oxidase, although the activity was considerably greater with the former substrate present (Figure 3). Control reaction mixtures containing no substrate or a heat inactivated particulate fraction showed no measurable activity. The inhibiting effects of cyanide and Antimycin A on the DPNH oxidase system are shown in Figure 4; TPNH oxidase was similarly affected by these inhibitors.

Figure 5 shows the DPNH and TPNH cytochrome c reductase activities of the particulate fraction. Again, activity was considerably greater in the presence of DPNH. There was no measurable activity in the absence of DPNH and TPNH or in a complete mixture containing a heat inactivated particulate fraction. Cytochrome c reductase activity was not inhibited by Antimycin A (Figure 5). The reduction of cytochrome c by succinate was also demonstrated (Figure 6); this activity was inhibited by Antimycin A, $5\text{ }\mu\text{g}/3.0\text{ ml. final volume}$.

Table 1. *The effect of benzoquinone on DPNH oxidase and DPNH cytochrome c reductase activities of a particulate fraction from Rumex virus tumor tissue.* For the measurement of DPNH oxidase activity the reaction mixture contained DPNH, 5×10^{-5} M; particulate suspension, 0.2 ml.; and, where indicated, p-benzoquinone, 2×10^{-4} M; 2,4-dinitrophenol (DNP), 10^{-5} M; Antimycin A, 5 μ g; sufficient buffer to make 3.0 ml.; pH 7.0; temp. 26°C. For measurement of DPNH cytochrome c-reductase, reaction mixture contained cytochrome c, 3×10^{-5} M; KCN, 10^{-3} M; DPNH, as above; particulate suspension, 0.1 ml.; buffer to make 3.0 ml. Reaction time, 5 mins. — Activity given as change in optical density.

Reaction mixture	DPNH-Cytochrome c reductase	DPNH oxidase
1) enzyme (boiled) + DPNH	.005	.01
2) 1 + benzoquinone01	.07
3) enzyme (active) + DPNH	.055	.10
4) 3 + benzoquinone025	.25
5) 4 + Antimycin A	—	.25
6) 4 + DNP	—	.25

TPNH and DPNH diaphorases were also shown to be active in the particulate cell fraction (Figure 7); the activity in the presence of DPNH being considerably greater than in the presence of TPNH.

The oxidation of DPNH was enhanced by the addition of benzoquinone to the reaction mixture; this enhanced oxidation was not affected by Antimycin A or 2,4 dinitrophenol (Table 1). The addition of benzoquinone appeared to have an inhibitory effect on the reduction of cytochrome c by DPNH (Table 1).

Discussion

Spectrophotometric evidence has been presented for the operation of a number of enzymes of the electron transport system in a particulate fraction from *Rumex virus tumor* cells. These particles, like all plant particles investigated to date (1) possess a cytochrome c oxidase. The relative activity of the enzyme appears to vary with the age of the tissue, a maximum rate occurring in 4-week old tissue. A variation in rate of oxygen uptake with age of tissue slices has been reported (6) but the age at which the maximum rate of oxygen consumption occurs does not coincide with the maximum rate of cytochrome oxidase activity, the latter appearing in somewhat older tissue. Attempts to demonstrate the cytochrome bands in extracts of the particles were unsuccessful because sufficiently large amounts of tissue could not be made available for this purpose.

Although both DPNH and TPNH oxidase activity is demonstrated by the particulate fraction, the latter substrate is oxidized at a very low rate. All

available evidence indicates that TPNH oxidase activity of plant particulate fractions is very limited (1). The DPNH oxidase activity of these particles, like this activity in plant particles in general (1), is inhibited by cyanide and Antimycin A.

DPNH and TPNH cytochrome c reductase activity has also been demonstrated here, but these reactions are insensitive to Antimycin A. Cytochrome c reductases which are insensitive to Antimycin A have been reported from plant tissues (1) and from other sources (7) although the role of these enzymes in the electron transport chain is not certain. The presence of a number of cytochrome c reducing systems makes it difficult to determine the role of any reductase associated with a particular cell fraction in the electron transport chain. In systems containing an Antimycin A-sensitive cytochrome c reductase, evidence has been presented for the presence of an Antimycin A insensitive pathway for DPNH oxidation (8). Further, Antimycin A has been shown to be ineffective as an inhibitor of soluble DPNH cytochrome c reductase in marked contrast to its effect on the same activity associated with particles (9). Succinate-cytochrome c reductase activity of the particulate fraction was also demonstrated; this activity is completely inhibited by Antimycin A. The particulate fraction also contains a DPNH and TPNH diaphorase.

It has been suggested that a quinone reductase may operate in the oxidation of DPNH (10). In the presence of p-benzoquinone, DPNH oxidase activity was increased; this increase was not inhibited by 2,4 dinitrophenol or Antimycin A. The quinone reductase system of pea seeds was inhibited as much as 95 % by 2,4 dinitrophenol, 10^{-5} M (10, 11). The failure of 2,4 dinitrophenol to inhibit this enzyme has been reported (12) and it has been suggested that, in such a case, the reduction of p-benzoquinone by DPNH may not be catalyzed by a specific quinone reductase but results from a nonspecific reaction of the enzyme diaphorase (12). Although p-benzoquinone has been reported to increase the rate of cytochrome c reduction by DPNH (12) it proved to be an inhibitor of the system used in these experiments.

Summary

Spectrophotometric analysis of a particulate fraction from cells of *Rumex* virus tumor tissue showed the presence of cytochrome oxidase, DPNH, TPNH, and succinate cytochrome c reductases, DPNH and TPNH oxidases, and DPNH and TPNH diaphorases.

Antimycin A inhibited DPNH and TPNH oxidase activity and the reduc-

tion of cytochrome c by succinate. DPNH and TPNH cytochrome c reductase activity was not affected by Antimycin A.

The DPNH oxidase activity was enhanced by the addition of p-benzoquinone. This increased activity was not affected by Antimycin A or 2,4-dinitrophenol.

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Studies of the Growth in Culture of Excised Wheat Roots

I. The Growth Effects of an Acid-Hydrolysed Casein and of Light

By

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Introduction

Root tips from maize and wheat seedlings were used in the pioneer attempts to grow excised roots (Kotte 1922, Robbins, 1922) and White (1932) also used wheat in his first studies on root culture. These cereal tips showed a high initial rate of growth on transference to the culture medium but this declined sharply after several days and the newly formed root axis progressively decreased in diameter. The duration of growth of the cultures was clearly limited and could not be maintained by subculture or by transference of the cultures to new culture medium.

Subsequently, there have been two reports of the successful establishment of excised cereal roots in continuous culture. McClary (1940) reported the growth of excised maize roots through at least eighteen transfers without progressive decline in their growth rate, though attempts to repeat this work have proved unsuccessful (Bonner and Bonner 1948, Robbins 1951). Roberts and Street (1955) reported that clones of excised roots of Petkus II winter rye could be established in continuous culture from 10 per cent of the seedlings of their grain sample and a confirmatory report on the culturability of rye roots has been published by Almqvist (1957).

Roberts and Street (1955) considered that their successful establishment of rye root clones followed from the addition of yeast extract (or tryptophane) to their standard root culture medium and the adoption of an appropriate subculture technique. This led them to postulate that the failure of

roots of most cereals and of many other plants to grow in sterile culture arose more from the special nutrient requirements of such roots than from injury effects involved in excision of the root tips, unfavourable physical conditions of culture or the accumulation of toxic metabolic products in the nutrient medium. The apparent importance of the duration of growth intervening between successive subcultures and the length of the root tips transferred at each subculture also suggested that a nutritive interrelationship between the meristematic and mature tissues might be important for the continuation of cell divisions in the excised root tips.

These considerations have guided the present re-examination of the behaviour in culture of excised root tips of wheat.

Materials and Methods

Mercury-dressed grain of the 1957 and 1959 harvests of Atson Elite 56 wheat (Plant Breeding Institute, Weibullsholm, Landskrona, Sweden) was sterilised by being immersed for 1 min. in 1 % Lissapol (Imperial Chemical Industries Ltd.), transferred to 0.1 % aqueous mercuric chloride (100 ml. per 200 grains) for 20 min. and then washed four times, each for 1 min. in 100 ml. sterile distilled water. The sterile grains were then transferred, 10 grains per dish, to sterile 9 cm. petri dishes each containing two Whatman No. 1 filter papers moistened with ca. 3.5 ml. sterile water and set to germinate in the dark at 27°C for 48–60 hr. Ten mm. root tips were excised from radicles 20–30 mm. long and transferred individually, under aseptic conditions, to the culture flasks. Satisfactory root tips have well developed root hairs and float on the surface of the culture medium; any tips which immediately sank in the medium were rejected. The rate of germination and initial vigour of excised root growth were influenced by the year of harvest of the grain and by the duration of its storage in the laboratory.

The standard culture flasks were 100 ml. wide-mouthed Pyrex Erlenmeyer flasks, closed with non-absorbent cotton plugs wrapped in gauze and protected by an inverted 50 ml. aluminium beaker. Each flask contained 50 ml. of culture medium. In most experiments the cultures were incubated at 25°C for 14 days. To study the persistence of growth this period was either extended without subculture or roots were subcultured by excision of their main axis tips (10 mm.) and transference to fresh medium. The successive culture periods involved in any one such experiment are termed passages and are numbered in sequence.

'Light' cultures were illuminated by overhead banks of mixed fluorescent (Ekco 80 watt, white) and tungsten (Osram 75 watt) tubes; each bank 3' × 5' was made up of 12 fluorescent tubes interspersed with 6 tungsten tubes. These banks gave, at the surface of the culture solution, an intensity of about 900 lux. Light intensities were measured with a Megatron type 'C' light meter. 'Dark' cultures were flasks covered with aluminium foil interspersed among the light cultures or where all cultures were 'dark', the cultures were incubated in a dark temperature-controlled room.

Linear growth of the roots in culture was recorded as the increase in main root

axis length (mm.) (I.M.A.), number of emergent laterals (L.N.) and total length of such laterals (mm.) (L.L.). The values quoted are means of at least 10 replicate cultures. Dry weights were obtained by bulking the roots of each treatment, rinsing them in distilled water, blotting and drying for 12 hr. at 70°C. Standard errors of growth values are not quoted in Tables and Figures but attention is drawn in the text only to significant growth effects. As an indication of the variability encountered within treatments, decreases or increases in excess of 25 per cent for values for I.M.A., of 35 per cent for L.N. and of 75 per cent for L.L. are found to be significant at the 0.05 level.

The basic root culture medium was a modified White's medium containing copper and molybdenum (Street and McGregor 1952), ferric sodium ethylenediamine tetraacetate (Sheat, Fletcher and Street 1959) and, as carbon source, 2 % D(+)glucose. The acid hydrolysed casein was Bacto-casamino Acids (B230) of Difco Ltd. Except in the examination of various sugars for their activity as carbon sources and in initial tests with Bacto-casamino Acids, the whole medium was sterilised by autoclaving at 15 lb./in² for 5 min. The alternative method of preparing the sterile medium was to autoclave the selected constituent separately and dissolved in water to one-fifth of the volume of the final medium and then to add this to the remainder of the medium constituents in four-fifths of the finished volume.

Where necessary, the pH of the medium was adjusted by the addition of dilute sodium hydroxide or hydrochloric acid.

Results

D(+)-Glucose as carbon source

Preliminary experiments, not now to be reported in detail, involved examination of D(+)-glucose, D(-)-fructose and sucrose as carbon sources. Glucose was superior to fructose or sucrose for all criteria of growth. Many of the roots, developed with sucrose as carbon source, showed within 14 days of culture complete loss of apical dominance, emergent laterals appearing right up to the tip of the main axis and many of the earlier formed laterals showing secondary laterals. It was, therefore, not surprising that when the ability of the main axis root tip to grow on subculture was tested the superiority of glucose over sucrose was particularly emphasised.

Provided the initial pH of the medium was carefully standardised the growth-promoting activity of glucose was not impaired by autoclaving it with the other medium constituents. The optimum level of glucose was, for roots grown in the basic culture medium, between 3 and 4 %, and for roots grown in presence of Bacto-casamino Acids (400 mg./l.) between 2 and 3 %. The optimum glucose concentration was shifted towards the lower values of these ranges by illumination of the cultures. These considerations prompted the adoption of 2 % glucose as the standard carbon source.

Standardisation of the initial pH of medium at 4.6-4.8

The basic medium had, after autoclaving, a natural pH of 4.1-4.2. This value was raised by additions of Bacto-casamino Acids. Tests with basic medium and with this supplemented by the Bacto-casamino Acids showed that lateral initiation and extension were progressively reduced as the initial pH of the medium was raised from 4.8 to 6.8. Because one of the primary objectives of the present work was to establish excised root clones for which lateral initiation and growth are essential, the initial pH of the medium was 4.6-4.8 except where otherwise stated. Subsequent experiments involving subculture through excision of main axis tips also showed that the duration of growth was reduced at pH values above 4.8.

The growth-promoting effects of Bacto-casamino Acids and light

Table 1 shows that the addition of Bacto-casamino Acids significantly enhances all aspects of the growth of 'dark' cultures. An even more marked enhancement of growth occurs when the cultures are illuminated. The addition of Bacto-casamino Acids to the medium of 'light' cultures, whilst having no significant or, in some experiments, a retarding influence on main axis extension growth always increases lateral initiation, lateral extension and dry weight production. The concentration of 400 mg./l. Bacto-casamino Acids was shown in a separate experiment to be a fully effective concentration for promotion of the growth of 'dark' and 'light' cultures.

Figures 1 and 2 present data from an experiment in which the roots were subcultured every 7 days or allowed to continue growth without subculture for 28 days. 'Dark' cultures in basic medium ceased growth after 14 days and total growth was reduced by subculture, cultures receiving Bacto-casamino Acids when subcultured grew through three passages and when

Table 1. *Growth of excised wheat roots in basic culture medium and in this medium supplemented by the addition of 400 mg./l. Bacto-casamino Acids, in darkness and when illuminated. Growth over 14 days at 25°C.*

Treatment		Growth values			
		I.M.A.	L.N.	L.L.	Dry wt. mg./15 roots
'DARK'	Basic medium	94	10.6	63	9.9
	Basic medium + Bactocasamino Acids	112	25	304	17.9
'LIGHT'	Basic medium	181	17.7	223	59.2
	Basic medium + Bactocasamino Acids	171	33.3	527	84.3

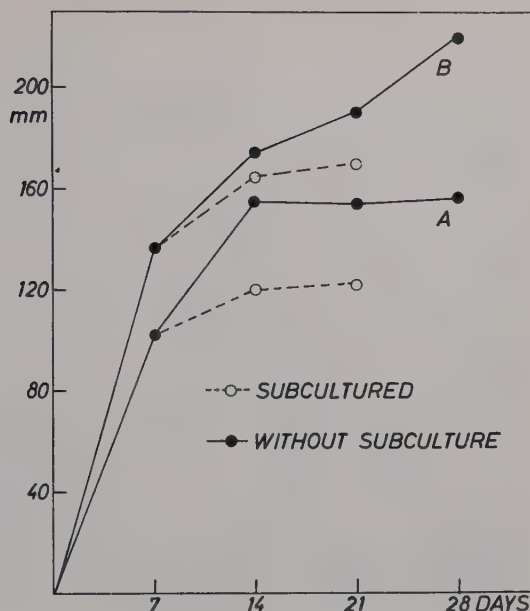


Figure 1. Values for total main axis increase (mm.) of 'dark' cultures in basic medium (A) and basic medium (pH 6.8) + Bactocasamino Acids (400 mg./l.) (B) over 28 days at 27°C.

not subcultured continued growth for the 28 days of the test. These results again indicate the beneficial effect of the Bacto-casamino Acids on growth. They also suggested that subculture hastens the depletion of some factor essential for the continuing growth of the roots.

Bacto-casamino Acids and light may enhance root growth in culture by

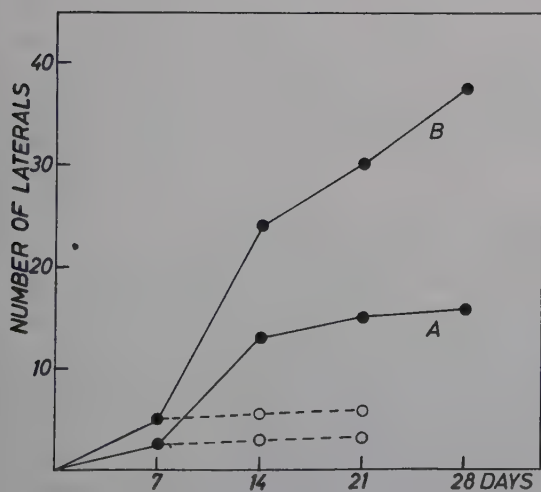


Figure 2. Values for total numbers of laterals of 'dark' cultures in basic medium (A) and basic medium (pH 6.8) + Bactocasamino Acids (400 mg./l.) (B) over 28 days at 27°C. Key as figure 1.

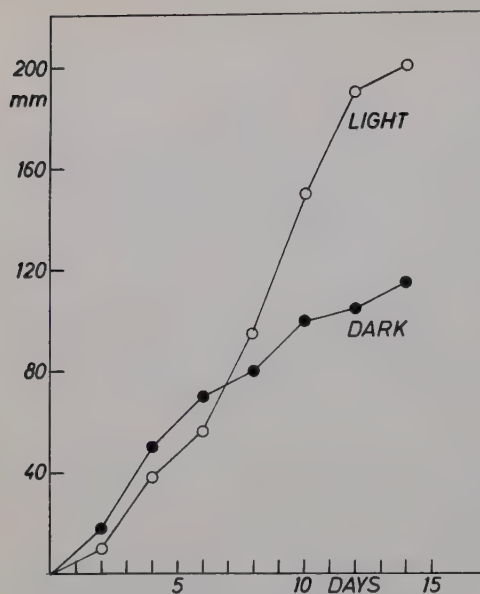


Figure 3. Mean values for increase in main axis length at intervals during the first 14 days of culture at 25°C in basic medium + Bactocasamino Acids (400 mg./l.)

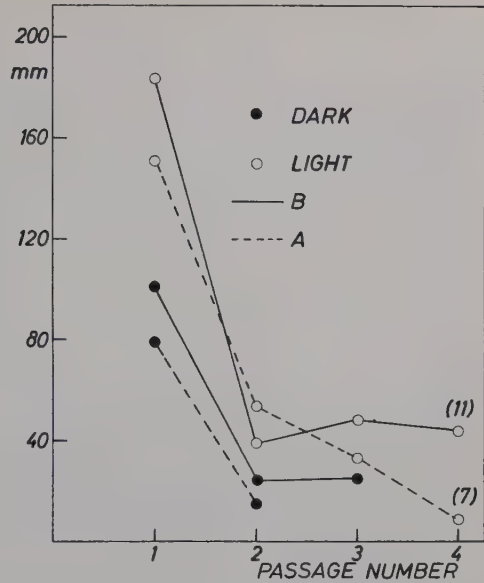
supplying directly or promoting the synthesis in the cultures of essential substances which the developing radicle normally receives from the endosperm but which are depleted as the excised root tip grows in culture. If this is so it might be expected that the promotion of growth by the Bactocasamino Acids and light would be most marked when the depletion of material carried over from the grain begins to limit growth. Study of the time course of the effect of light on cultures receiving Bactocasamino Acids conforms to this prediction. Figure 3, which is a plot of the increase in main axis length of 'light' and 'dark' cultures, shows that light, initially inhibitory to growth, becomes after the 6th day of culture very stimulatory and permits growth to continue after it has almost ceased in the 'dark' cultures.

Experiments involving repeated subculture

The experiments described above indicated that the duration of the growth of the root cultures was prolonged by addition of Bactocasamino Acids and by illumination. The next step, therefore, was to determine the full extent of the duration of meristematic activity when the roots were repeatedly subcultured in presence of these cultural treatments

The results of an experiment involving four successive 14 day passages are shown in Figures 4 and 5. 'Dark' cultures ceased growth in basic medium

Figure 4. Main axis increase (mm.) in four successive passages (14 days) of roots cultured in light and darkness and in basic medium (A) and in this medium + Bactocasamino Acids (400 mg./l.) (B). Sixteen cultures for each treatment were initiated; the numbers in brackets show the numbers of growing cultures in the 4th passage for the light cultures.



in the second, and in presence of Bacto-casamino Acids in the third passage. Some of the 'light' cultures of both treatments grew in the fourth passage. The addition of Bacto-casamino Acids not only enhanced growth in each passage but also the proportion of the initial cultures still growing in the fourth passage. These observations selected treatments involving both addi-

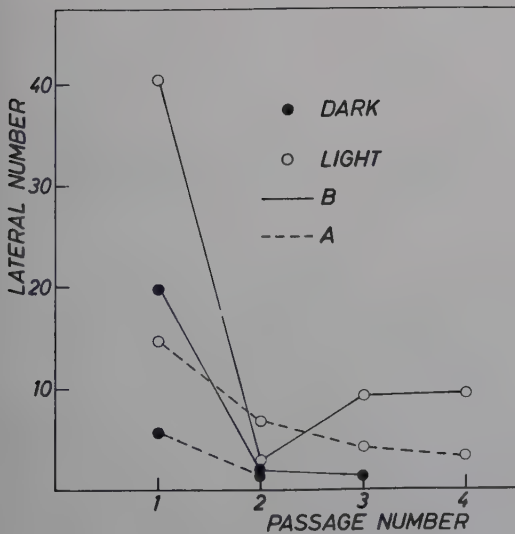


Figure 5. Mean lateral number per root for the four successive passages of the experiment detailed in the legend to Figure 4.

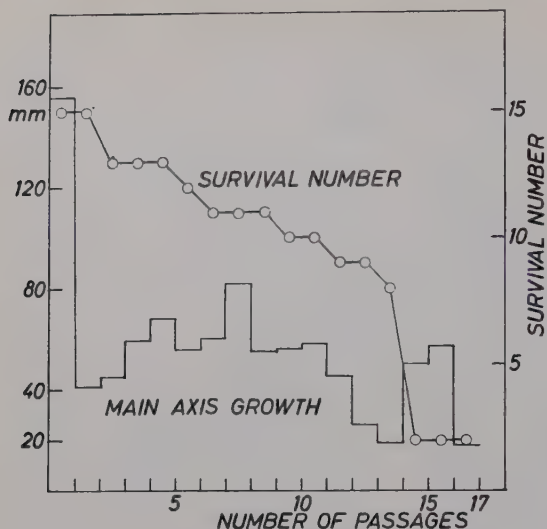


Figure 6. Main axis growth values (mm.) and number of growing cultures in each passage (survival numbers) for each of 17 successive 14-day passages for roots of illuminated cultures in basic medium + Bactocasamino Acids (400 mg./l.).

tion of Bacto-casamino Acids and illumination for a longer term experiment. The observations of Roberts (1954) on the importance of the passage length for the successful culture of rye roots suggested this as the additional variable in such an experiment.

The long term experiment extended over 40 weeks and examined, using a medium containing 400 mg./l. Bacto-casamino Acids and the standard conditions of illumination:

- (i) the growth of roots subcultured by excision of 10 mm. main axis tips every 7, or 14, or 21, or 28 days.
- (ii) the growth of roots subcultured every 14 days by excision of main axis tips of 10 and 30 mm. lengths.
- (iii) the growth of laterals on isolated segments ('sectors') cut from the main axes of root cultures at the end of the first and selected subsequent passages.

In the main part of this experiment (section 1, above) a small number of cultures was still growing, at each passage length, after 30 weeks. The initial high rate of growth declined markedly after the first 14 days of culture and from then onwards a low and, from passage to passage, very variable growth rate was maintained. This is illustrated in Figure 6 which shows the mean values for main axis increase in successive passages for the roots subcultured every 14 days. During the period of continuing low growth rate individual cultures completely ceased growth and were not subcultured.

Table 2. *Total growth of excised wheat roots cultured in basic medium + Bactocasamino Acids (400 mg./l.) and illuminated, over 24 weeks.*

Summed mean growth values from successive passages	Passage length (interval between successive subcultures by excision of 10 mm. main axis tips)			
	7 days (24 Passages)	14 days (12 Passages)	21 days (8 Passages)	28 days (6 Passages)
Main axis increase (mm.).....	711	780	617	691
Number of laterals	145	176	140	122

The number of cultures (initially 15 in each treatment) surviving, therefore, declined progressively and when the experiment was terminated no treatment was represented by more than 3 cultures. The outstanding feature of this part of the experiment was that passage length did not have a decisive effect upon the duration of growth. Nevertheless, higher 'survival numbers' (Street, McGonagle and Roberts 1953) did occur in those subcultured every 14 days. More than 50 % of the initial cultures continued growth for 28 weeks when subcultured every 14 days as against 8, 6 and 4 weeks with subculture periods of 7, 21 and 28 days, respectively. Furthermore, somewhat more total growth occurred under the regime of 14 day passages than with other passage lengths as shown in Table 2 where the summed mean growth values for all passages over the first 24 weeks of the experiment are compared.

The use of 30 mm. instead of the standard 10 mm. root tip for subculture each 14 days (section (ii) above) did not significantly affect the mean growth values recorded but had a depressing effect upon 'survival numbers'. When cultured in fresh medium, sectors (10 mm.) of the main root axis, bearing 4 or 5 emergent laterals, always showed some extension growth of their laterals (section (iii) above). This extension of the laterals proceeded slowly for at least 4 weeks. Neither the rate nor extent of this lateral growth was significantly affected by the location along the root axis at which the sector was excised. Sectors excised at intervals during the first eight successive 14-day passages showed very similar extents of lateral growth. The ability of the excised tips of these laterals to grow in culture was not affected by the passage at which the parent 'sector' culture was initiated. The growth of these lateral tips was variable and not significantly different from that of the low level of growth of the parent cultures (the level established 14 days after establishment from the seedling radicle tip). After the eighth 14-day passage, however, lateral initiation in the cultures was so poor that 'sector' cultures could not be established.

Discussion

The very significant enhancement of the growth of excised wheat roots resulting from addition of Bacto-casamino Acids to the culture medium and illumination of the cultures supports the view that basic root culture media do not meet the special nutrient requirements of these roots. That these two factors are apparently not able to prevent a marked decline in the growth of the cultures with time, a decline hastened by subculture, however, strongly suggests that some additional growth requirement of the roots remains to be discovered. The long periods over which a low level of root growth can be maintained in presence of Bacto-casamino Acids and light may perhaps indicate that the roots do synthesise, under these conditions, their other essential nutrients but that one or more of these remain at a critically low level limiting growth and rendering the persistence of growth on subculture precarious. The second 14-day passage of our wheat roots provides us with a very sensitive test for this as yet hypothetical factor limiting growth.

Clearly, no claim can be made that conditions for the continuous culture of excised wheat roots have been achieved until the cultures grow, over a prolonged period, at a level more nearly approaching that observed during the first 14-days of culture. Before searching for the additional growth factor postulated above, it seems, however, important to study in greater detail the growth-promoting activities of the Bacto-casamino Acids and light. The hydrolysed casein by its very complexity may not present its active constituents under the most favourable conditions, and a complex light spectrum has been used in the present experiments at a quite arbitrary intensity.

Summary

The addition of Bacto-casamino Acids (400 mg./l.) to the culture medium and illumination of the cultures (900 lux, fluorescent light) enhances both the level and duration of growth of excised roots of Atson Elite 56 wheat. These factors do not prevent, after 14 days of culture, the occurrence of a marked decline in growth rate and neither satisfactory continuous culture nor the establishment of clones has been achieved.

We wish to express our indebtedness to Dr. J. D. Ferguson and Professor G. A. Gries who, while in our Department during the session 1957-8, participated in the exploratory work leading to the choice of the wheat variety, Atson, and to the testing of casein hydrolysates. Thanks are also expressed to the Nuffield Foundation for their support, particularly by the provision of a Research Fellowship (J.D.F. and subsequently E.G.S.) and two Research Assistantships (J.E.C. and D.S.).

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Stem Cutting and Water Movement in Young Barley Plants

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It has been shown in previous studies that severing the water-conducting tissues of the plants cause changes in the rate of transpiration. Thus cutting of the stem induces a brief increase in transpiration, followed by a decrease (Darwin 1898, 1904, Iwanof 1928, Rufelt *et al.* 1954, Allerup 1959). If the stomata of plants treated in this way are kept under observation, the openings are seen to widen transiently. The brief widening is followed by a closing action. The movements of the guard cells are found to correspond in time to the transpiration changes (Darwin and Pertz 1912, Laidlaw and Knight 1916, Knight 1922, Allerup 1960). Based on this accordance in time Allerup (1960) explained the causal relationship between the observations by assuming that the cutting releases the tension to which the water phase within the plant is subjected during transpiration. In this way the water supply to the leaves is suddenly but transiently improved, the guard cells are able to take up water, and the stomata are widened. The conditions for release of water vapour from the leaves are momentarily improved, and transpiration increases. However, since uptake of water by the plant is made impossible by the cutting, the increased transpiration is maintained only for a short period of time, after which the stomata are closing and transpiration decreases.

In order to examine the validity of the above theory, it was considered necessary to prove that the cutting does in fact induce a rupture of cohesive forces in the water phase of the plant, causing part of the water to move towards the leaves and reach these sufficiently rapidly. In the following an attempt to support the theory in this way is described.

Methods

A number of methods have been used in studies concerning the translocation of water in plants, and a common feature of these methods appears to be the labelling of the transported water. The labelling technique varies; heat is used in some cases, as in the thermoelectric method (Huber 1956, and others), or else molecules of an easily recognizable tracer is introduced locally in the water phase of the plants. A vast number of substances have been used as tracers over the years, *e.g.*, lithium salts (Sachs 1878), dyes (Preston 1952, and others), fluorochromes (Strugger 1939), radioactive isotopes (Ladefoged 1960, and others), and also the heavy oxygen isotope O^{18} (Vartapetyan and Kursanov 1959).

In the present study we used a radioactive isotope (P^{32}) both in order to make the investigations as objective as possible, which is difficult when dyes or fluorochromes are used, and also in order to select a tracer which is demonstrable in extremely small quantities. It was important to be able to follow both the changes in the rate of water flow and the changes in the distribution of the water. An additional advantage involved in the use of this tracer is that it becomes possible to cultivate the plants in a salt solution of the same chemical composition as that used for the experiments; this of course would be quite impossible if, for instance, lithium salts were used. On the other hand, the use of P^{32} introduces other sources of errors since the phosphorus atoms (in this case in an orthophosphate) can hardly be expected to follow the path of the water molecules under all conditions. However, for the brief experimental periods used here (1 second to 30 minutes) P^{32} is not likely to participate in the metabolic processes to an extent compromising the results. In fact, a number of investigations concerning the applicability of the method have shown P^{32} to be a most appropriate tracer in the type of experiment here described.

The use of H_2O^{18} might appear preferable, but it should be remembered that the molecular weight of the tracer would then become higher than that of the ordinary water molecules by about 10 per cent; this difference might cause the tracer molecules to assume a flow rate different from that of the ordinary water molecules within the plant. Also, density measurements of non-radioactive isotopes is very demanding with regard to apparatus.

Sample plants were 6 to 10 days old barley seedlings (Carlsberg variety) cultivated as described by Allerup (1960). Prior to the experiments the plants were removed from the plastic slab, and damaged plants or plants suspected of having been damaged were immediately discarded.

The radioactive solution used was made up from 1.0 ml of a solution declared by the supplier (Radiochemical Laboratories, Amersham) to contain 1.8 mc P^{32} in the form of H_3PO_4 . This solution was mixed with 100 ml of an ordinary nutrient solution prepared according to Olsen (1950), and the resulting mixture was used for all of the present experiments. Due to the natural decay the activity of P^{32} was reduced over the period of the experiments to a maximum value of 0.65 mc. At the beginning of an experiment, the lower parts of the plants were placed in the radioactive solution. If the plants were to remain intact throughout the experiment, all of the root and half of the seed were submerged in the radioactive solution; plants to be cut were placed in such a way that the possibility of cutting immediately below the surface of the liquid was ensured. All cuttings were made immediately following submersion. The onset of the experiment is considered to be the moment of submersion, respectively cutting, in the radioactive solution.

During the experiments the plants were kept in position by means of a piece of split rubber tubing held by a stand mounted spring clamp. The plant is in no way squeezed by the rubber tubing but is merely suspended within the cavity formed.

The plant is removed from the solution at the end of the experimental period. The parts which have been submerged are wiped off carefully but quickly between layers of paper tissue, and the plant is then placed on a sheet of graph paper. The above-ground parts of the plant (*i.e.*, stem and leaves) were cut by means of a razor blade into 2 cm long sections counted from the seed or from the cut (in a few experiments 1 cm sections were used). Severance starts at the top and works towards the root which is consequently, in the case of intact plants, severed last and discarded. The plant sections are then mounted on labelled, ordinary microscopic slides by means of scotch tape in such a way that when the slide is placed in the counting assembly the plant section is directly opposite the center of the window of the GM tube.

The counting assembly used was a Baird-Atomic Multiscaler II, model 132, with a GM-tube made by NCA, model 1 WAA, its window having a mass thickness of 1.55 mg/cm². The GM tube was placed in iron housing of a minimum thickness of 10 cm. The background for the assembly was 18 cpm. The functioning of the counting assembly was controlled daily by means of a uranium standard. Throughout the experiments it was attempted to maintain a natural counting error of about 3 per cent.

During the experiments the treatment of the plants was varied in the way described, one half of the samples being kept intact while the other half had their stems cut immediately above the seed at the beginning of the experiment. In addition, the transpiration conditions were varied. Thus one half of the experiments were carried out with plants whose leaves during the experiment were surrounded by slowly flowing air of a relative humidity of about 40 per cent, *i.e.*, relatively dry air. For the remaining half of the experiments the leaves were placed in stationary air, almost saturated with water vapor.

Results and Comments

Figure 1 shows a representative selection of the results obtained. Cutting is seen to induce a strong increase in the rate of water flow, radioactivity being found throughout the stems and leaves even after a period of only about 15 seconds after cutting, whereas in intact plants nearly 20 minutes are required for the activity to be equally extensive. An additional fact not to be seen from the figure is that the total amounts of radiophosphorus taken up by the intact plants are much lower (by a factor of about 10) than those found following cutting.

When interpreting the curves it is necessary to keep in mind that the lower part of the stems of the intact plants has hardly been in contact with the radioactive solution, whereas the cut plants have been sufficiently deep in the liquid to allow cutting to take place below the surface. Hence some of the radioactive solution may have adhered to the plants, thus causing abnormally high activity values in the lower part of the cut stems. In the figure this would

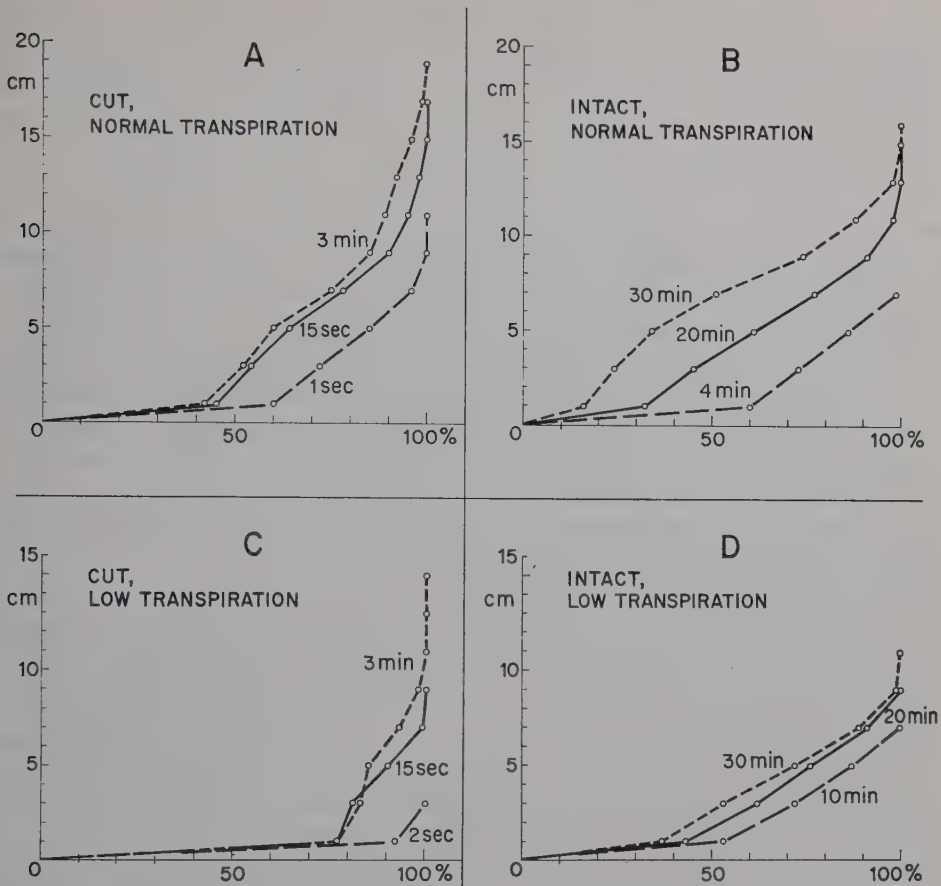


Figure 1. *Distribution of radioactivity in stems and leaves of barley plants following uptake of radiophosphorus under different conditions. The ordinates represent the distance between the plant sections used for activity countings and the neck of the root (intact plants) or the place of incision. The axes of abscissas show the percentage of the total radioactivity of the plants contained in a sample section and in the sections below it; hence 100 per cent represents the upper limit of distribution of radioactivity. The figures against the curves show duration of the experiments. A: cut plants, leaves in slowly flowing air of a relative humidity of 40 %. B: intact plants, transpiration conditions as in A. C: cut plants, leaves in stationary, water saturated air. D: intact plants, transpiration conditions as in C.*

show up by the lower parts of the curves being levelled off and shifted towards the right. Furthermore, caused by the basis of calculation chosen, the adhered activity in the lower parts may reduce the percentage of activity found in the upper leaves, thus also in this respect contributing to an erratic course of the curves. Thus the shapes of the curves cannot be compared

without reservations. Curves representing different samples may be compared only with the utmost care, while curves from the same sample may be compared somewhat more easily.

Whether the experiments are made under conditions allowing only slight transpiration (leaves in air saturated with water vapor) or under more normal conditions, they show cutting to induce a sharp increase in the flow rate of water, manifested by a distribution of activity throughout the plant after only a brief interval, *i.e.*, about 15 seconds in dry air and about 3 minutes in humid air (compare curves *A* with *B* and *C* with *D*). It may also be noted that plants having their leaves in saturated air as expected show much lower total activity than do plants kept under conditions allowing greater transpiration.

Hence the experimental facts strongly support the theoretical considerations about the effect of cutting on transpiration. It seems well established that the cutting releases a tension in the unbroken water phase within the plant, allowing a more abundant flow of water towards the leaves. Thus a transient increase in transpiration is made possible, all the more since the richer water supply causes the stomata to open.

The presence of a continuous water phase within the plants was also demonstrated in a number of experiments in which the transpiration flow was reversed. The longest leaf of each plant was lacerated at the tip (in a length of about 1 cm) by means of a razor blade, and subsequently placed in the radioactive solution. If the root was left in air, radioactivity was present throughout the stem and leaves after 5 minutes, and after an additional 15 minutes throughout the root as well. If on the other hand the transpiration of the root was hampered by placing it in a nutrient solution, a period of 15 minutes was required before all of the water in the leaves was found labelled, and an additional period of about 1 hour was needed before it was possible to trace radioactivity in most of the root. Thus the continuous water phase within the plants is more or less easily moved in one direction or the other, according to which parts of the plant dominate with regard to transpiration and consequently induce a pull in a certain direction.

Summary

The fact that the water phase of the plants is continuous and subjected to tension caused by transpiration has been proved by labelling the water taken up with P^{32} . The tension may be released by cutting the stem. Severance induces an increased supply of water to the leaves, manifested by the rapidity with which radioactivity is distributed after cutting the stem below the surface of a liquid containing P^{32} . Under normal transpiration conditions P^{32} may

be traced throughout the entire plant 15 seconds after cutting, while radio-activity deposited via the ordinary transpiration flow in the intact plant can be demonstrated with certainty in the upper parts of the leaves only after about 20 minutes.

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Orcylalanin, seine Verteilung und physiologische Bedeutung in *Agrostemma githago* L.

Von

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Die Kornrade (*Agrostemma githago* L.) enthält das 2,4-Dihydroxy-6-methylphenylalanin, eine aromatische Aminosäure, die aus den Samen isoliert werden konnte (Schneider, 1957, 1958, 1958). Die Benzolringsubstitution der Aminosäure gleicht der des Orcins. Deshalb ist für sie die Bezeichnung Orcylalanin gewählt worden. Das Orcylalanin weicht durch seinen Orcinkern wesentlich von der Struktur der bisher bekannten aromatischen Aminosäuren ab. Bisher konnte sein Vorkommen nur in der Kornrade sichergestellt werden. Die chemische Struktur und die Tatsache, daß die neue Verbindung nicht als Baustein der *Agrostemma*-Proteine erscheint, lassen vermuten, daß sie im Stickstoffhaushalt der Kornrade eine von den anderen aromatischen Aminosäuren abweichende Bedeutung hat. Diese wurde durch Ermittlung der Orcylalaninverteilung in den Pflanzenteilen der Kornrade und die Analyse des Metabolismus der Aminosäure im Verlauf der Pflanzenentwicklung untersucht. Die Gegenüberstellung der Ergebnisse mit den entsprechenden Daten des Gesamt-, Protein-, löslichen und Amid-Stickstoffs sollen eine Einschätzung der Funktion des Orcylalanins im Stickstoffhaushalt der Kornrade ermöglichen.

Methoden

Vorbereitung des Materials. Die frischen Pflanzenteile wurden zur Abtötung mit einem Äther-Alkohol-Gemisch (1 + 1) benetzt, in abgedeckter Schale auf dem kochenden Wasserbade erhitzt, nach Verdunstung des Äthers und Alkohols 3-4

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Minuten in Siedehitze belassen und alsdann im Vakuum über Schwefelsäure bei 40°C getrocknet. Das trockene, fein gemahlene Pflanzenpulver wurde über Schwefelsäure aufbewahrt.

Stickstoffbestimmungen. Die Gesamtstickstoffwerte wurden nach der üblichen Kjeldahl-Methodik durch Veraschung von je 50 mg Pflanzenpulver mit 5 ml konzentrierter Schwefelsäure und Titration des im Alkalischen überdestillierten Ammoniaks mit 0,02 N Lösungen erhalten. — Die Proteinstickstoffbestimmungen erfolgten nach Extraktion des Materials mit 2,5 %iger Trichloressigsäurelösung bei Zimmertemperatur während 14 Stunden in gleicher Weise wie beim Gesamt-N. Der Gehalt an löslichen Stickstoffverbindungen ergab sich aus der N-Differenz dieser beiden Fraktionen. Von den in 2,5 %iger Trichloressigsäure löslichen Verbindungen wurde der Stickstoffgehalt der Amide (einschließlich des Ammoniak-N) ermittelt. Ein aliquoter Teil des Extraktes wurde drei Stunden in 1 n Schwefelsäure auf dem Wasserbade hydrolysiert und alsdann das freigewordene Ammoniak bei pH 12 vorsichtig in 0,02 N Schwefelsäure überdestilliert. — Die Ermittlung des Orcylalaningehaltes erfolgte durch Colorimetrie des gelben Farbstoffes, der nach Behandlung der Aminosäure mit diazotierter Sulfanilsäure in saurem Milieu auftritt. Ein mit Natronlauge neutralisierter, aliquoter Teil des Trichloressigsäureextraktes, der 0,2–2,5 µg Orcylalanin/ml enthielt und auf 10 ml mit Wasser verdünnt war, wurde mit 3 ml diazotierter Sulfanilsäure (25 ml 1 %ige Sulfanilsäurelösung + 5 ml Natriumnitritlösung, 5 %ig, + 2,5 ml Essigsäure + 17,5 ml destilliertes Wasser) versetzt und nach 30–60 Minuten die optische Dichte der entstandenen Gelbfärbung im Spektralphotometer bei 410 mµ und 2 cm Schichtdicke gemessen. Als Standard diente eine 10⁻⁵ molare Orcylalaninlösung. Diese Bestimmungsmethode wird durch Anwesenheit anderer diazotierbarer Aminosäuren nicht gestört. Ihre Meßgenauigkeit beträgt ± 5 %.

Ergebnisse

a) Die Orcylalaninverteilung in den Geweben der Kornrade

Das Orcylalanin ist in der vollentwickelten, fruchtenden Pflanze in geringen Mengen vorhanden. Es verteilt sich auf die Pflanzenorgane nicht

Tabelle 1. Der Orcylalaningehalt einiger Organe von *Agrostemma githago* zur Zeit des Fruchtens.

Organ	Orcylalanin × 100	Orcylalanin-N × 100
	Frischgewicht	Gesamt-N
Embryonen	1,490	1,39
Samen	0,452	1,06
Blüten	0,096	0,83
Knospen	0,065	0,57
Blätter	0,053	0,50
Kelche	0,037	0,37
Kapseln	0,029	0,45
Sproßachse	0,016	0,29
Wurzeln	0,004	0,13

gleichmäßig. Den höchsten Gehalt erreicht es in den Samen und ist hier vornehmlich in den Embryonen, die 1,49 % vom Frischgewicht enthalten, zu finden. Etwa ein Achtel des Orcylalaningehaltes der Samen ist in den Blättern, 1/25 in der Sproßachse und 1/100 in den Wurzeln vorhanden. Der Anteil des Orcylalaninstickstoffs am Gesamtstickstoff liegt in den Samen bei 1 %, in den Knospen und Blättern erreicht er die Hälfte und in der Sproßachse ein Drittel dieser Konzentration (Tab. 1).

b) Die Verteilung des Orcylalanins im Verlauf des Pflanzenwachstums

Die zur Untersuchung benutzten Keimlinge der ersten 3 Entwicklungstage wurden in Petrischalen auf Fließpapier, das mit destilliertem Wasser durchfeuchtet war, bei 20°C im Dunkeln angezogen. Ältere Pflanzen waren in Sand kultiviert worden. Sie wurden im Wechsel mit Knoop'scher Nährlösung und destilliertem Wasser begossen und standen im Gewächshaus. — In den

Tabelle 2. Der Gehalt an löslichem N, Orcylalanin-N und Amid-N im Verlauf der Entwicklung von *Agrostemma*-Pflanzen in % vom Trockengewicht. In Klammern: % vom löslichen Stickstoff.

Stadium und Pflanzenteil	lösl.N	Orcylalanin-N	Amid-N
Samen, ruhend	0,58	0,030 (5,2)	0,056 (9,7)
Keimlinge nach:			
1 Tag	0,71	0,029 (4,1)	0,060 (8,4)
2 Tagen	0,73	0,029 (4,1)	0,074 (10,1)
3 Tagen	0,86	0,033 (3,8)	0,187 (21,7)
Kotyledonen nach:			
1 Tag	1,86	0,097 (5,2)	0,147 (7,9)
2 Tagen	1,76	0,097 (5,5)	—
3 Tagen	1,41	0,058 (4,1)	0,156 (11,1)
Sprosse nach:			
7 Tagen ¹	1,19	0,040 (3,3)	0,140 (11,8)
14 Tagen ²	0,61	0,020 (3,3)	0,107 (16,3)
Wurzeln nach:			
1 Tag	2,27	0,071 (3,1)	0,218 (9,6)
2 Tagen	1,88	0,034 (1,8)	0,412 (22,0)
3 Tagen	1,53	0,016 (1,1)	0,432 (28,2)
34 Tagen ³	0,27	0,004 (1,5)	0,040 (14,8)
110 Tagen ⁴	0,24	0,001 (0,5)	0,030 (13,1)

¹ Die Pflanzen waren im Mittel 4 cm groß.

² Die Pflanzen waren im Mittel 10 cm groß.

³ Die Pflanzen waren im Mittel 13 cm groß und hatten 2-3 Blattpaare.

⁴ Freilandpflanzen in Vollblüte.

ersten 3 Entwicklungstagen bleibt in den Keimlingen und Keimblättern der Anteil des Orcylalanins an den Verbindungen, die zum löslichen Stickstoff zählen, konstant; in den Wurzeln fällt er in der gleichen Zeit auf ein Drittel der Konzentration am ersten Tage. Der Stickstoff der Amid-Fraktion erhöht in gleichem Zeitraum seinen Anteil am löslichen Stickstoff in den Keimlingen und Wurzeln um das 2–3fache. In geringerem Maße wächst sein Anteil in den Keimblättern. In den Wurzeln sinkt mit dem Altern der Pflanze der Anteil des Orcylalanins und des Amid-N am löslichen Stickstoff gleichartig vom dritten Tage bis nach 110 Tagen auf etwa die Hälfte ab (Tab. 2).

c) *Der Orcylalaningehalt reifender Samen von Agrostemma githago*

Im jüngsten Reifestadium, in dem die Samen noch weiß und in eine schleimige Flüssigkeit innerhalb der Samenkapsel eingebettet sind, ist noch kein Orcylalanin nachweisbar. Bei fortschreitender Reifung verliert das Kapselinnere die Schleimflüssigkeit und die noch weißen Samen erreichen nahezu ihre endgültige Größe. Zu diesem Zeitpunkt sind die ersten Spuren von Orcylalanin aufzufinden. Die Orcylalaninmenge nimmt nun stetig zu und erreicht ihren höchsten Wert, wenn die Samen zur Vollreife gelangt sind. Der Gehalt an löslichen Stickstoffverbindungen und der der Amide (mit Ausnahme des Amidgehaltes in weißen Samen) unterliegen im Verlauf der Samenreife geringen Schwankungen. In der Tabelle 3 sind die Reifestadien der Samen durch ihre Verfärbung, das Trockengewicht, Gesamtstickstoff- und Proteinstickstoff-Gehalt charakterisiert.

Tabelle 3. *Trockengewicht und Stickstoffbilanz reifender Samen von Agrostemma githago.*
N-Gehalt bezogen auf das Trockengewicht.

Reifestadium	% Trocken- gewicht	Gesamt-N	Protein-N	Lösl-N	Orcyl- alanin-N	Amid-N
weiß	37,3	2,08	1,46	0,62	0,005	0,157
hellbraun	42,7	2,28	1,66	0,62	0,007	0,067
dunkelbraun	46,8	2,48	1,84	0,64	0,012	0,059
schwarz 1	51,8	2,60	2,00	0,60	0,017	0,070
schwarz 2	55,5	2,78	2,21	0,57	0,024	0,066
schwarz 3	87,5	3,04	2,35	0,69	0,027	0,050

d) *Der Orcylalaningehalt notreifender Samen von Agrostemma githago*

Als Versuchsmaterial dienten Kapseln von Freilandpflanzen, die in 10-tägigen Abständen geerntet wurden und zum ersten Erntetermin weiße, zum

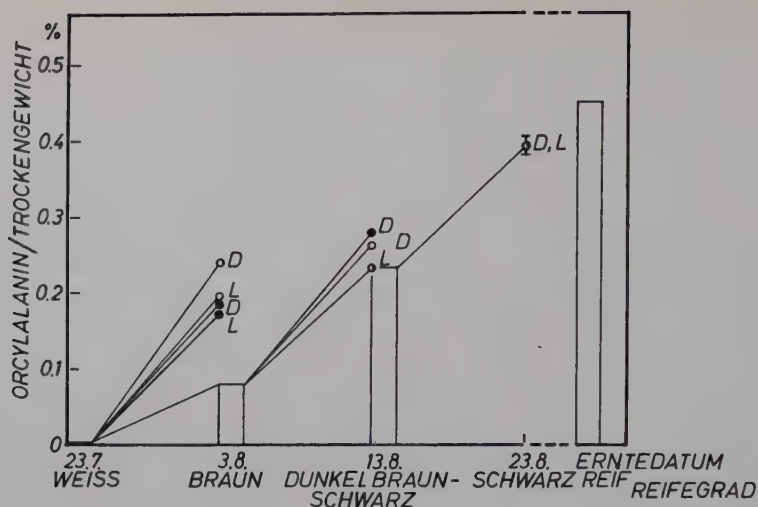


Abbildung 1. Orcylalanin Gehalt reifender und notreifender Samen von *Agrostemma githago*. L. ○ = Kapseln — ● = Sprosse. L = Lichtaufenthalt — D = Dunkelaufenthalt.

zweiten Termin braune und schließlich dunkelbraunschwarze Samen enthielten. Von jeder Ernte wurden zwei Versuchsreihen angesetzt. In der einen wurden die Kapseln am beblätterten Sproß, der etwa 20 cm über dem Erdboden abgeschnitten worden war und von dem die älteren 2–3 Blätter entfernt wurden, in Wasser 10 Tage im Versuchsgewächshaus bzw. in der Dunkelkammer bei 20°C gehalten. In einer zweiten Serie wurden vom Sproß getrennte Samenkapseln 10 Tage bei etwa 20°C im Licht eines nach Norden weisenden Fensters bzw. im Dunkeln gelagert. In den auf diese Weise notgereiften Samen wurde der Orcylalanin Gehalt ermittelt und mit dem von Samen, die am natürlichen Standort gereift waren, verglichen. Die notreifenden Samen, die zu Versuchsbeginn weiß waren und noch kein Orcylalanin enthielten, sind nach 10 Tagen bedeutend orcylalaninreicher als die entsprechenden Freilandkontrollen. In den Samen isoliert gelagerter Kapseln übersteigt die Orcylalaninkonzentration um das 2,5–3fache und in den Samen der am Sproß verbliebenen Kapseln etwa um das 2fache die der Kontrollen. Zur Erntezeit braune Samen haben nach der Notreifezeit im Licht den gleichen Orcylalanin Gehalt wie die Kontrollen; im Dunkelaufenthalt liegt er um 11 % bzw. 18 % über den Vergleichswerten. Die dunkelbraun-schwarzen Samen erreichen während des Versuches, unabhängig von der Behandlungsweise, 80–90 % des Orcylalanin Gehaltes vollstündig ausgereifter Samen (Abb. 1).

Diskussion

Die Verteilung des Orcylalanins ist in den Geweben der Kornrade recht unterschiedlich. Während die Wurzeln vollentwickelter Pflanzen praktisch orcyalaninfrei sind, ist in den Blütenorganen und insbesondere in den Samen eine bemerkenswerte Anreicherung der Aminosäure zu beobachten. Die Samen sind offenbar das Speicherorgan der Verbindung, die vornehmlich in den Embryonen lokalisiert ist. Diese bevorzugte Anreicherung des Orcylalanins in den Samen ließ vermuten, daß die Aminosäure eine Stickstoffspeicherverbindung sei. Die in den Tabellen 1 und 3 aufgeführten Untersuchungsergebnisse stützen jedoch diese Annahme nicht. In dem Orcylalanin sind nur 1,1 % des Gesamtstickstoffgehaltes der Samen festgelegt, während 1,6 % auf die zum Vergleich herangezogenen Amide und 77 % des Stickstoffs der Samen auf die Proteine entfallen. Die Analyse einzelner Reifestadien der Samen zeigt zudem, daß der Gehalt an löslichen Stickstoffverbindungen im Verlauf der Reifeperiode nahezu konstant bleibt und der Proteingehalt in dem Maße zunimmt wie der Gesamt-N steigt. Nach diesen Befunden speichern die *Agrostemma*-Samen ihren Stickstoff als Reserveprotein. Das Orcylalanin und die Amide sind für diese Funktion weitgehend bedeutungslos. Das schließt nicht aus, daß das Orcylalanin bei der Samenkeimung eine besondere Stoffwechselfunktion innehat. Um eine solche zu erkennen, wurden die Orcylalanin- und Amid-Konzentration im Verlauf der Pflanzenentwicklung bestimmt und die Ergebnisse gegenübergestellt. Der Orcylalaningehalt bleibt in den ersten Entwicklungstagen der Pflanzen unverändert. Dagegen steigt die Amidmenge in den Keimlingen um das Dreifache an und deckt nahezu die gesamte Stickstoffzunahme in der Fraktion der löslichen Stickstoffverbindungen (Tab. 2). Das beweist, daß im Zuge der Reserveproteinmobilisierung in keimenden Samen fast nur Amide entstehen. Dem Orcylalanin kann somit auch in diesem Bereich des Stickstoffhaushaltes der Kornrade keine Bedeutung zukommen. — Erst mit fortschreitender Entwicklung sinkt die Orcylalaninkonzentration in den Geweben der Kornrade ab. In reifenden Samen ist schließlich wieder eine Konzentrationszunahme der Aminosäure zu beobachten (Tab. 3). Um zu klären, ob das Orcylalanin in vegetativen Organen der Kornrade gebildet und mit dem Nährstoffstrom in die Samen translociert wird oder ob die Aminosäure direkt in den Samen entsteht, wurden die Notreifeversuche angestellt. Nach den Befunden über den Verlauf der Orcylalaninsynthese in isoliert gelagerten Kapseln entsteht die Aminosäure hauptsächlich in den Samen- oder Kapselgeweben. Die Synthese ist somit nicht abhängig vom Zustrom spezifischer Verbindungen aus dem Sproß. Die Intensität der Orcylalaninsynthese ist in den Samen der vom Sproß getrennten Kapseln größer als in den Samen, deren Kapseln am

Sproß verblieben waren. Auch liegt der Orcylalanin Gehalt der Dunkelvarianten stets höher als der der entsprechenden Lichtvarianten. Wahrscheinlich ist die beobachtete verstärkte Orcylalaninzunahme in den Samen der im Dunkeln gehaltenen Versuchspflanzen gegenüber den Parallelen im Licht relativ, da die Trockenmasse pro Samen infolge der Hemmung der Kohlenhydratspeicherung im Dunkeln in geringerem Ausmaße zunimmt als im Licht. Die absolute Zunahme an Orcylalanin kann demnach in den Samen der Lichtreihen größer sein als in denen der Dunkelreihen. Schließlich lassen die unterschiedlichen Voraussetzungen zur Kohlenhydratspeicherung in den Samen der im Licht gehaltenen Kapseln und Sprosse die Annahme zu, daß die Samen der Sproßkapseln — absolut betrachtet — etwas mehr Orcylalanin bilden als die der isolierten Kapseln. Diese durch die Bezugsgröße (Trockengewicht) bedingten Abweichungen von der wahren Orcylalaninverteilung in den Samen beeinträchtigen die Schlußfolgerungen der Untersuchungen nicht, denn wichtig ist allein, daß in den Samen isoliert gelagerter Kapseln auch dann Orcylalanin entsteht, wenn ihre Entwicklung durch einen empfindlichen Mangel an organischen Baustoffen gekennzeichnet ist. Die Bildung der Aminosäure steht danach in enger kausaler Abhängigkeit vom Reifungsprozeß der Samen. Sie kann nicht durch ungünstige Umweltbedingungen blockiert werden. Das deutet eine wichtige physiologische Funktion der Aminosäure an, die bisher nicht erkannt wurde. Es ist aber wenig zweifelhaft, daß der Aminosäure in der Fraktion der löslichen Stickstoffverbindungen eine untergeordnete Bedeutung zukommt, wie sie etwa den aromatischen Aminosäuren Phenylalanin und Tyrosin zuzusprechen ist. Das Orcylalanin gehört aber, im Gegensatz zum Tyrosin und Phenylalanin, nicht zu den Aminosäuren, die als Proteinbausteine zumeist nur in spärlicher Menge in der Fraktion der löslichen Stickstoffverbindungen anzutreffen sind. Die Aminosäure scheint, wenn sie eine wesentliche Funktion ausübt, außerhalb des N-Stoffwechselbereiches zu wirken. Im Stickstoffhaushalt ist ihr Metabolismus etwa mit dem stickstoffhaltiger sekundärer Pflanzenstoffe vergleichbar.

Zusammenfassung

Es wurden die Orcylalaninverteilung in den Organen von *Agrostemma githago* im Verlauf der Entwicklung, der Syntheseort der Aminosäure und die Bedeutung der Verbindung im Stickstoffhaushalt der Pflanze analysiert. Die Wurzeln sind der orcylalaninärmste, die Samen der orcylalaninreichste Pflanzenteil der Kornrade. Die Aminosäure, die in den Embryonen lokalisiert ist, wird im Verlauf der Fruchtreife gebildet. Ihre Synthese ist nicht von der Zufuhr spezifischer Vorstufen aus dem Sproß abhängig. Das Orcylalanin hat

weder bei der Samenreife noch bei der Keimung die Funktion einer Stickstofftranslocations- bzw. Stickstoffspeicherverbindung. Sein Metabolismus ist für die N-Bilanz im Verlauf der Pflanzenentwicklung von untergeordneter Bedeutung.

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Effect of Soil Moisture Content on the Cell Wall Metabolism of Sunflower and Almond Leaves

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In earlier studies on *Avena* coleoptile sections it was found that moisture stress inhibited incorporation of C^{14} from glucose- C^{14} into various components of the cell wall (11, 12). Cellulose synthesis appeared to be of greatest interest since both growth and incorporation into cellulose responded similarly to turgor changes in the tissue. An extension of this investigation to other plants, particularly intact ones, was required. Furthermore it is necessary to know the effect of irrigation history on cell wall metabolism. This object was achieved by growing sunflower and almond shoots in pots under different soil moisture regimes.

Two main problems were clarified:

1. What was the effect of soil moisture regime history on the glucose- C^{14} incorporation, added when plants from various irrigation treatments were at a uniform soil moisture content (at field capacity).
2. What was the effect of the instantaneous soil moisture content at the time of the addition of the glucose, on its incorporation.

In the case of sunflower plants, the work was accordingly divided into two series of experiments:

Series A: Examination of the effect of the moisture regime history.

Series B: Examination of the combination of the moisture regime history with the instantaneous situation. Through a comparison of Series A and B it is possible to determine the effect of the instantaneous moisture situation alone.

Only the influence of the soil moisture regime history was examined in the almond shoots.

Materials and Methods

Sunflower plants, *Helianthus annuus* L., and almond shoots, *Prunus amygdalus* Batsch, were grown in cylindrical metal pots which contained 800 g. and 4.5 kg. (calculated on a dry weight basis) of soil respectively. The soil used for the experiments was a sandy loam taken from Gilat (in the northern Negev). This soil, which had a field capacity of 18 % and a wilting point of 7.5 %, was prepared in a standard manner, i.e. crushed to pass through a 2 mm. sieve. Four to five seeds of sunflower or almond were sown in each soil-filled pot. The pots were fertilized with nutrient solution which contained N, P, K, in accordance with the recommendations of Jenny *et al.* (7). All experiments, except one, were conducted in a glasshouse where the temperature and humidity were not controlled. Temperature during C¹⁴ incubation is given with data. The second experiment of Series B was conducted in a growth chamber at a constant temperature of 26°C and 12-hour daily illumination by fluorescent lamps (1,000 foot candles). In all of the experiments, the plants were distributed on tables according to a completely randomized design.

The pots were irrigated daily with enough water so that the moisture content within each pot was relatively uniform a short time after irrigation. This moisture content was 24 % by weight. Uniform irrigation conditions prevailed until the beginning of differential treatments. At this stage the roots filled the entire volume of the pot. This point occurred in the sunflower when the plant had two pairs of leaves, and in the almond when the plant reached a height of 18 to 20 cm. The plants were thinned to one plant per pot, selection being made to increase the uniformity of the experimental plants.

Four irrigation treatments were then established, in each of which the moisture was reduced to a different level before irrigation was applied. The treatments are described in Table 1. Differential treatments continued until the driest treatment reached its minimal point once for Series A sunflowers and Series B experiment 1 and twice for almonds and Series B Experiment 2.

Uptake of glucose-C¹⁴ was accomplished by making a vertical slanting cut in the stem to create a xylem containing ligule which was immersed in a small vial containing 0.1 ml. of uniformly labeled glucose with a specific activity of 5 µc/0.1 ml. The absorption of the solution began in the morning, and during the day the vial was rinsed with 0.1 ml. of water. In Series A all plants were irrigated to field capa-

Table 1. Summary of irrigation treatments.

Treatment	Pre-irrigation moisture content	Pre-irrigation moisture tension atm.
1	7-8 %	15
2	13 %	1
3	18 %	0.3
4	22 %	0.2

city 18 hours prior to the addition of glucose- C^{14} . In Series B, glucose- C^{14} was administered while the plants were at the lowest soil moisture characteristic of a given treatment. After 8 hours of C^{14} uptake the plants were cut and the leaves and stem were weighed separately. Three replications were used in the first experiment of Series B, and six replications in all of the other experiments. The leaves were frozen in liquid air and were stored in a freezer compartment. The stems were dried at 70°C , to determine dry matter.

Dry weight was determined on replications not exposed to C^{14} . Three replications of each treatment were used to determine the diffusion pressure deficit (DPD) in the leaf tissues, according to Ursprung's method (17).

The leaves in which cell wall metabolism was investigated were dried by lyophilization and over P_2O_5 . The dried material was weighed and ground. A weighed aliquot was ground in a glass mortar in ice water to separate the cell wall from the cytoplasm. The sunflower leaf homogenate was filtered through a sintered-glass plate funnel (filtrate = A_1). Further fractionation was similar to that reported earlier (11). The residue was extracted in sequence with boiling 95 % ethanol, hot water, hot 0.05 N HCl, 5 % KOH, 24 % KOH and acetolyzing reagent ("hot" means temperature when vessel immersed in boiling water bath). The extracts are designated as fractions A_2 , B, C, D, E and F respectively.

After the extraction of each fraction the residue was dried in the funnel with ethanol and ether, stored overnight in a dessicator over P_2O_5 , and weighed. In this manner it was possible to determine the weight of the material extracted.

Fractionation of the almond leaves was carried out by the same method, except that the extracts were separated from the residue by centrifuging at 1,000 g. for 10 minutes.

Samples of fractions A, B and C were subjected to hydrolysis according to Bishop's method (1) and the component sugars were separated by two dimensional paper chromatography, in water-saturated phenol, and in butanol:acetic acid:water (4:1:5). The various sugars were identified by position after development with a spray of aniline phthalate solution. The carbohydrates of fractions D, E were precipitated with ethanol (8) and the precipitate was subjected to hydrolysis and chromatography as above. Fraction F was first subjected to hydrolysis by acid and the residual acetic acid was removed by steam distillation; excess sulfate was precipitated by barium carbonate (8) and the supernatant solution was chromatographed. To determine the radioactivity of the separated sugars, the appropriate spots were cut out of the chromatogram and were counted on planchets.

From each extract of known volume an aliquot quantity of the solution was placed on stainless steel planchets. The water, alcohol and acid soluble fractions and the cellulose were dried directly at low temperature under a lamp. The alkaline extracts were first neutralized with hydrochloric acid. The radioactive counts of these latter extracts and of the cellulose fraction (F) were corrected for self-absorption. The samples were counted by means of a gas-flow counter fitted with a micro-mil window in an atmosphere of Q-gas. The duration of the count was such as to achieve a precision of 3 %.

If C_{fi} is the total radioactivity of fraction "i" in a leaf sample, then:

$$\begin{aligned} i &= F \\ \sum C_{fi} &= \sum_i C_{fi} = \text{total activity.} \\ i &= A \end{aligned}$$

This calculation is based on the assumption that no material was lost during fractionation. All of the extracts were collected, except the ether extract, whose radioactivity was almost nil, and the residue that remained in the funnel, which was also very low in radioactivity. The data are presented as relative radioactivity, i.e. total activity of fraction as per cent of the total activity in the leaves.

Since the distribution of non-radioactive material was found to be equal in the cell wall from the different treatments (14), comparisons based on total radioactivity in the various fractions among the treatments can be made.

Results

Series A — Sunflowers

That the soil moisture regime influenced plant growth may be seen in Table 2, which presents data on dry weight of the leaves, stems and total tops (15 replications in each treatment).

Statistical analysis of the differences between the various treatments shows that in the dry weight of the leaves, stems and total tops, there was a significant difference at the 1 % level between treatments 1 and 2, and between treatments 3 and 4; between treatments 2 and 3 no significant difference was found.

DPD analysis of the leaves showed that the irrigation given prior to uptake of radioactive glucose eliminated differences in DPD in the plants of the various treatments.

The total activities, ΣC_{fi} , were approximately equal. The values for treatments 1 through 4 were 1,120, 1,093, 1,067 and 998×10^3 cpm respectively. Although these figures show a certain trend the differences are not statistically significant.

The relative radioactivity of the various fractions is presented in Figure 1 (solid lines). The counts of the fractions soluble in ice-water and alcohol (A_1 , A_2) were combined into one fraction, A.

The data on fraction A shows that as the soil moisture regime during the period of growth prior to the C^{14} uptake period became drier, there was a

Table 2. *Dry weight of leaves, stems and total tops of sunflower plants grown under four different soil moisture regimes. Values in mg.*

Treatment	No. of irrigations	Leaves	Stems	Total Tops
1	1	189.9	258.6	448.5
2	2	263.9	317.6	581.5
3	4—5	281.6	318.2	599.8
4	18—20	342.4	364.4	706.8

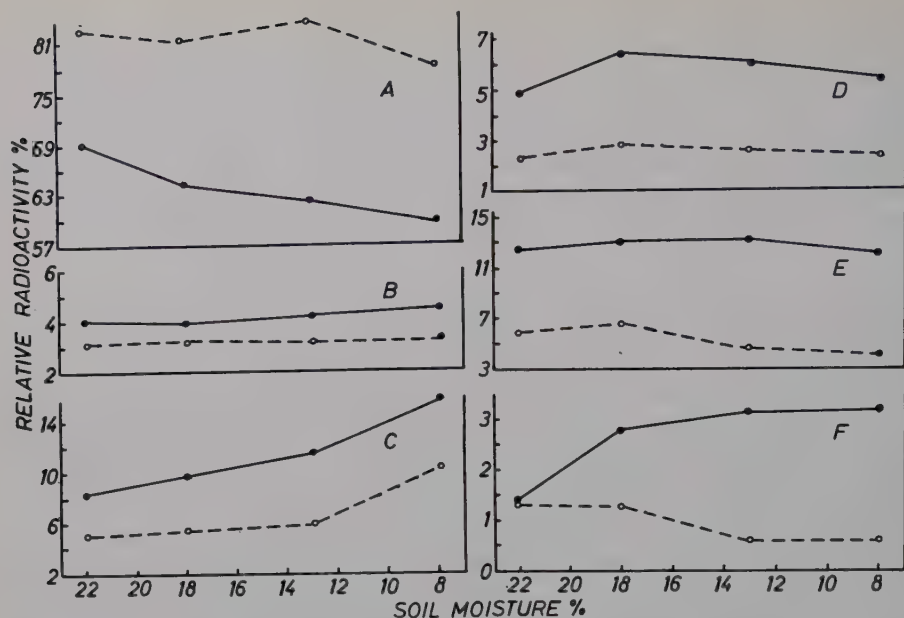


Figure 1. Relative radioactivity of various fractions of sunflower leaves (—) and of almond leaves (- - -). Plants were grown under four soil moisture regimes (Series A: soil moisture at field capacity during C^{14} incubation). Temperature during incubation for almonds = 23 to 36°C. Temperature during incubation for sunflowers = 12 to 27°C.

progressive reduction of radioactivity. A statistical analysis of the differences between the averages of the treatments showed all of them to be significant.

Fraction A was found to contain primarily glucose and relatively less arabinose and xylose. It was found that glucose was most active — 8 to 14 times more than each of the other sugars.

Apparently the high percentage of radioactivity in this fraction is due mainly to the free glucose that had reached the leaves but had not been incorporated. The lesser amount of free glucose found following drier moisture regimes shows that incorporation of glucose into the cell wall components was greater. It can be seen from Figure 1 that there was an increase only for fraction C and the cellulose fraction (F).

The drier the soil moisture regime was during the period prior to incorporation of C^{14} , the greater was subsequent incorporation into these two fractions. Statistical analysis of differences between the averages of the various treatments shows that in fraction C all the differences were significant; in fraction F the differences were significant, except between 8 %

moisture and 13 % moisture. On the other hand, no treatment effects were discernible in the other fractions.

The sugar composition of fractions B and C were similar. Both contained primarily glucose and somewhat less arabinose and xylose. They also contained two uronic acids which were not identified. Fraction D contained primarily xylose and to a smaller degree, arabinose. Only traces of glucose were found. This fraction also contained a uronic acid which was not identified. Fraction E contained an appreciable quantity of glucose, an intermediate quantity of arabinose and a smaller quantity of xylose. Only glucose was found in fraction F after hydrolysis.

Changes in radioactivity of the various sugars paralleled the changes in the fractions from which these sugars were isolated.

Series A — Almonds

The soil moisture regime also affected the growth of the almond plant as shown in Table 3 (six replications of each treatment).

Maintenance of a high moisture content decreasing only from 24 % to 22 % brought about a decrease in accumulation of dry material. Apparently this plant is sensitive to poor soil aeration. Statistical analysis shows that all of the differences were highly significant (1 % level).

In this experiment as well, ΣC_{fi} were similar for the various treatments. The average values found for treatments 1 through 4 were 1,653, 1,565, 1,663, $1,546 \times 10^3$ cpm respectively.

The relative radioactivity of the different fractions is presented in Figure 1 (broken lines).

Reduced relative radioactivity in fraction A was not as apparent in the dry treatments of almonds as it was in sunflowers. Even the difference between the driest and the wettest treatments was not significant.

The sunflower and the almond differ from one another in the degree of incorporation of C^{14} from glucose within the constituents of the cell wall.

Table 3. *Dry weight of leaves, stems and total tops of almond shoots grown under four soil moisture regimes. Values in g.*

Treatment	No. of Irrigations	Leaves	Stems	Total tops
1	2	1.66	1.82	3.48
2	4—5	2.45	2.87	5.32
3	12—15	5.01	6.30	11.31
4	45—47	3.13	3.26	6.39

In the almond the cell wall contained only 17 to 22 % of the total radioactivity while in sunflowers the cell wall contained 32 to 40 % of the total, *i.e.* the incorporation in sunflowers was almost twice as high.

Analysis of the cell wall constituents showed significant differences between the treatments for fraction C. The relative radioactivity was inverse to the soil moisture content during the growing period. This result was similar to that obtained with sunflowers. In cell wall fractions B, D, and E no significant differences were found. The results for these fractions were also similar to those obtained with sunflowers.

There was, however, a conspicuous difference between the two plants with respect to the influence of soil moisture regime history on incorporation of glucose-C¹⁴ into cellulose. In almonds grown under dry conditions there was a marked decrease in this incorporation.

The sugar composition of fraction A was similar to that in sunflowers. Again the main sugar was glucose, with an additional small quantity of sucrose and traces of arabinose and xylose.

Chromatographic examination of the cell wall fractions showed that in almonds there was a great similarity between fractions B and C. To a certain extent these fractions resembled the corresponding ones in sunflowers. Both fractions contained primarily glucose and arabinose after hydrolysis. Xylose was found in small quantities only. In addition, three uronic acids were found.

Fraction D contained, unlike sunflowers, primarily arabinose, glucose, and two uronic acids. Fraction E contained primarily xylose, glucose and traces of arabinose. In the cellulose fraction after hydrolysis of the extract, primarily glucose was found, although there were also traces of xylose. Thus the last three fractions differed in their composition from the corresponding sunflower fractions.

Series B — Sunflowers

The differences in the instantaneous moisture condition of the soil were accompanied by differences in DPD in the plant tissues, as shown in Table 4. We find here a fairly good correlation between the DPD in the leaf tissues and the moisture percentage in the soil.

In the first experiment of this series incorporation was examined in only two moisture treatments. In the dry treatment minimum moisture during the growth period (and at the time of incorporation as well) was 7 to 8 %. The resulting radioactivity in each fraction is presented in Table 5. In the second treatment the minimum moisture was 16 %

From Table 5 it is apparent that differences in soil moisture have a notable effect on the metabolism of the cell wall carbohydrates. Radioactivity

Table 4. *Diffusion pressure deficit in the leaves of sunflower as related to soil moisture content.*

Soil moisture content, %	DPD, atmospheres
7—8	17.0—18.0
9—10	14.0—15.0
12—13	9.5—10.5
15—16	6.5—7.5
18—19	4.5—5.5
21—22	3.0—4.0

was decreased in each cell wall fraction as a result of increased soil moisture stress. Higher relative radioactivity was obtained under conditions of high moisture tension in fraction A. Therefore, with an increase in tension, there was a decrease in the incorporation of C^{14} from glucose into the cell wall constituents.

In order to obtain a more complete picture of the effect of the entire range of available moisture on the incorporation of glucose into cell wall constituents, an additional experiment, containing four moisture treatments, was performed. These treatments were parallel to those of Series A.

The average ΣC_{fi} of treatments 1 through 4 (see Table 1) in this experiment were 996, 1,236, 1,379, $1,312 \times 10^3$ cpm respectively.

This experiment showed differences in the degree of absorption of radioactive glucose by the leaves. With a reduced DPD in the plant, in conjunction with increased soil moisture, there was in general an increase in the uptake of glucose.

The relative radioactivity data of each fraction are presented in Figure 2. This experiment confirmed the results of the previous experiment. The decrease in radioactivity of the cell wall constituents was conspicuous particularly when moisture content fell below 18 %. Statistical analysis of the differences between the treatments shows that below 18 % soil moisture

Table 5. *Relative radioactivity (%) in fractions of sunflower leaves grown under various soil moisture regimes* (Radioactive glucose was introduced when the soil moisture was at the minimum for each treatment, Series B Experiment 1). Temperature during C^{14} uptake and incubation 18° to 23°C.

Treatment	Fractions					
	A	B	C	D	E	F
Dry	74.13	3.27	4.85	7.54	9.02	1.33
Moist	56.35	4.30	9.07	12.90	14.50	2.69

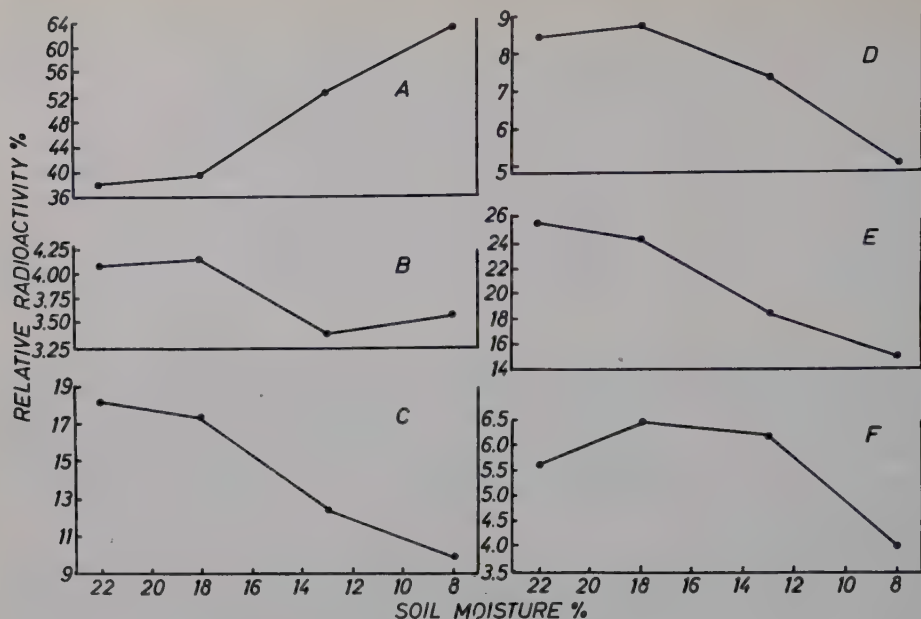


Figure 2. Relative radioactivity of various fractions of sunflower leaves. Plants were grown under four soil moisture regimes (Series B, Experiment 2: soil moisture at minimum point for each treatment during C^{14} incubation). Temperature during incubation = 26°C .

there was a significant decrease in incorporation, while a change in the range between 18 to 22 % moisture did not cause a significant change in the incorporation of glucose- C^{14} into any of the cell wall fractions. A partial explanation of this may be derived from the moisture retention data of the soil in which the plants were grown (Table 1). Apparently the slight rise in

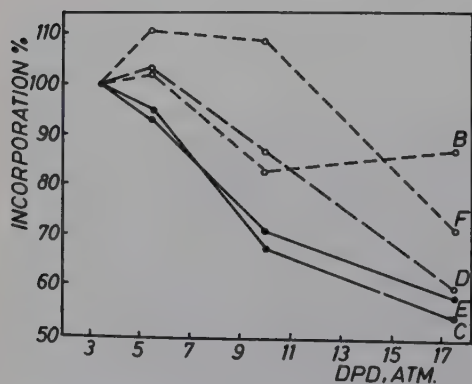


Figure 3. Relation between incorporation of glucose- C^{14} into cell wall constituents of sunflower leaves and DPD of leaf tissue. Conditions of figure 2. On the ordinate incorporation as per cent of wettest treatment.

tension from 22 % moisture to 18 % moisture did not cause a significant difference in the availability of the water for certain processes, such as synthesis of the cell wall constituents.

The incorporation into each fraction in the various treatments can also be compared by setting the wettest treatment at 100 % in each case, and comparing the other treatments with it. Such results are presented in Figure 3 where the incorporation is plotted as a function of tissue DPD.

It appears that above a certain level of DPD (about 5 atmospheres) there was an almost linear relationship between increase of DPD and decrease in incorporation into most constituents (C, D, E, F).

Discussion

The results of Series A of this investigation clearly points to the conclusion that the history of the soil moisture regime affected primarily the synthesis of the acid-soluble fraction and cellulose. Synthesis of other cell wall fractions was not affected by the soil moisture regime history when either sunflower or almond soils were at field capacity during the C^{14} incubation. This effect of soil moisture history may be explained as a response of certain synthetic processes to prior soil moisture regimes after the reduction of DPD and its equalization in all treatments. This could be demonstrated by a comparison of the behavior of sunflower in Series A with that in Series B.

The results of Series A may be considered as a response to variations which were nullified, and the results of Series B primarily as a response to instantaneous moisture condition and partly as an effect resulting from previous history.

One may suggest two explanations for the response to instantaneous moisture differences:

a) Synthesis of new cell wall matter by cytoplasm may be limited as a result of reduced turgor pressure (*i.e.* less cell wall cytoplasm interaction).

b) Osmotic pressure per se in the plant tissue may inhibit certain enzyme systems directly or affect the contact between the enzyme and its substrate.

Although it is not known how cytoplasmic pressure on the cell wall facilitates deposition of new cell wall matter, Schaefer (16) indicated that contact between wall and cytoplasm decreases in the hypotonic region as incipient plasmolysis is approached. It is clear that under conditions of incipient plasmolysis, new cell wall matter would cease to be deposited as a result of breaking of contact.

In connection with the second explanation, the sensitivity to osmotic pres-

sure of the different enzyme systems associated with synthesis of wall polysaccharides is unknown.

The fact that irrigation eliminated any apparent influence of moisture regime history in all the treatments for fractions B, D, and E agrees with the results on *Avena coleoptiles* (12) where it was shown that a rise in turgor caused a rise in incorporation of C^{14} into most of the cell wall components, compared with incorporation in coleoptiles which remained at low turgor.

The stimulation of synthesis of fraction C in sunflowers and almonds and of fraction F in sunflowers after irrigation following unfavorable moisture conditions appeared to compensate for the earlier retardation. If the formation of a block at one of the stages of synthesis, when the moisture tension was high, is postulated, a high concentration of intermediates might accumulate at that point. If we assume that the concentration of intermediates was rate limiting during optimal growth and that a reduction in moisture tension removes this block, the rate of synthesis of the final products may then be accelerated. Since all of the sugars in a given wall fraction appeared to be affected equally by moisture conditions, the effect must be on something common or prerequisite to the transfer of all of the sugars to the one or more acceptors involved.

The results of Series A are of interest in relation to certain conclusions of Gates (5, 6) who found higher net assimilation rates and growth rates in plants that were irrigated after reaching the wilting point, in comparison with plants grown at continuously high moisture content. Gates explained these results as a tendency of the plant to age physiologically with the reduction of moisture, and a tendency toward rejuvenation after irrigation.

The retarded synthesis of the cellulose fraction in almonds is difficult to explain because we do not know the effect of instantaneous moisture conditions on incorporation of glucose- C^{14} in almonds. Possibly there was very low incorporation into the cellulose of the almond during conditions of high tissue DPD and irrigation caused an increase in incorporation which nonetheless did not equal that of the moist treatments. On the other hand, the two plants may react differently in the recovery of synthetic activity after decrease of moisture tension, particularly in the case of cellulose.

Nezgevorova (10) arrived at results apparently different from ours. She showed that in short period experiments utilization of $C^{14}O_2$ by corn and oats for synthesis of hemicellulose and cellulose, increased in plants grown in dry soil compared with plants grown in wet soil. It is difficult to compare this data with our results of Series B since the moisture condition of the plants which were exposed to $C^{14}O_2$ is not known. If incorporation of the radioactive matter took place at the driest point in each treatment, there is a basic difference between our results and those of the aforementioned work.

It is possible that this would be caused by differences in the behavior of the different plants, or by the fact that plants were examined at different stages of synthesis. However, if incorporation took place soon after irrigation, than both the conditions and the results (10) were similar to those of our Series A.

Cell wall metabolism has been implicated in growth, particularly in the cell expansion phases. Pectin metabolism in coleoptiles has been found to be particularly affected by auxin (13) while there appears to be a further relationship between growth and cellulose which is not directly affected by auxin (12). Other workers (9, 15) suggested that hemicelluloses also play a part in growth. Since composition is variable from species to species and in different development stages (2, 4) different cell wall substances may have varying roles in cell expansion. Furthermore, as Burström (3) suggested, the structural pattern of cell wall deposition during growth may be of major importance rather than just the amount deposited. In any case, interference with the metabolism of these substances due to deficient moisture might be expected to contribute to reduced growth responses.

Summary

The effect of soil moisture content on the incorporation of glucose C^{14} into cell wall constituents of sunflower plants and almond shoots was investigated.

Both plants which had been grown under different soil moisture regimes and which were brought to field capacity before incorporation of glucose- C^{14} showed equal incorporation into certain cell wall components. In dilute acid-soluble fractions an increase in incorporation of C^{14} was found in the plants which had been grown under a dry soil moisture regime compared with plants grown in moisture close to field capacity during the entire growth period. A similar effect was found in the cellulose fraction of sunflowers. In almonds, however, which had been grown under a dry soil moisture regime prior to incorporation of glucose- C^{14} there was reduced incorporation into cellulose, compared with plants grown under a moist soil moisture regime.

Plants grown under a dry moisture regime and in which incorporation of C^{14} took place when the soil moisture of each treatment was at its minimum level, showed a reduction of incorporation into all fractions of the cell wall, compared with plants which during their entire growth period (including the time of incorporation of glucose- C^{14}) were at a soil moisture approaching field capacity.

An inverse relationship was found between the DPD in the plant tissues and the degree of incorporation into a number of the cell wall constituents.

In both plants, accumulation of dry matter in the various parts of the plant decreased with an increase in soil moisture tension of the irrigation regime.

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On the Effects of some Flavonoid Pigments upon Growth and Ion Absorption of Wheat Roots

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Introduction

Flavonoid compounds are widely distributed in tissues of higher plants (see Geissman and Hinreiner 1952, Stoll and Jucker 1958, Karrer 1958) and the chemical structure of a great variety of substances is well established. As regards their physiological effects in plants, on the other hand, relatively little is known. The interest has mainly been devoted to the anthocyanins, whereas the physiology of flavanone and flavone derivatives has not been studied to the same extent (see Geissman 1955, Blank 1958). These last-mentioned compounds may play some role in the enzymatic oxidation of phenols and ascorbic acid (see *e.g.* Huszák 1937, Blank 1958, Neumann *et al.* 1960) but the experimental results do not permit any definite conclusions. Moewus (1950) considered that flavones were physiologically active in the germination of pollen but his views have been doubted by *i.a.* Esser and Straub (1954). Recently Hendershott (Hendershott 1959, Hendershott and Walker 1959 a, b) drew attention to the flavanone derivative naringenin, which he suggested to be a naturally occurring inhibitor in peach flower buds. In view of this it seemed of interest to investigate its growth effects upon roots. As it proved active in tests with wheat roots its effects upon some other processes (respiration, ion absorption and sugar absorption) were also studied. In order to find out if the properties of naringenin were specific to this substance or common to many flavonoid pigments, some other flavones, isoflavones, and flavanones were included in the investigation. The following substances have been tested:

Pinocembrin (5,7-dihydroxyflavanone)
Pinobanksin (3,5,7-trihydroxyflavanone)
Naringenin (5,7,4'-trihydroxyflavanone)
Hesperetin (5,7,3'-trihydroxy-4'-methoxyflavanone)
Chrysin (5,7-dihydroxyflavone)
Apigenin (5,7,4'-trihydroxyflavone)
Morin (3,5,7,2',4'-pentahydroxyflavone)
Quercetin (3,5,7,3',4'-pentahydroxyflavone)
Genistein (5,7,4'-trihydroxyisoflavone)
Biochanin A (5,7-dihydroxy-4'-methoxyisoflavone)

For the generous gift of substances I am indebted to Professor Charles Mentzer, Paris (chrysin and apigenin), Professor Holger Erdtman, Stockholm (pinocembrin and pinobanksin) and Dr. Anna Nilsson, Uppsala (genistein and biochanin A). Hesperetin and naringenin were obtained from L. Light & Co., quercetin from Mann Research Lab., and morin from E. Merck, Darmstadt.

Experimental Results

The methods applied were essentially the same as those used in previous investigations (see Stenlid 1957 a, b, 1959 a, b). Seedling roots of spring wheat (Svalöf's Diamant II) were used. The growth experiments were performed with intact seedlings, the other experiments with excised roots.

The following abbreviations will be used: IAA for 3-indolylacetic acid, 2,4-D for 2,4-dichlorophenoxyacetic acid, 1-NMSP for α -(1-naphtylmethylsulfide)-propionic acid, 2-DG for 2-deoxy-D-glucose, and DNP for 2,4-dinitrophenol.

The effect of the flavonoid substances upon root growth is shown in Tables 1 to 5 and Figure 1. The flavonoids inhibit when applied in concentrations greater than a critical value, while lower concentrations often give stimulations. Especially pinobanksin and pinocembrin cause an obvious increase of root growth (20–40 %) in the concentration range 10^{-6} to 10^{-5} M. The effects resemble those reported for substances of anti-auxinic nature (see Torrey 1956, Åberg 1957) and suggest the possibility that the flavonoids act as auxin antagonists. Growth tests were therefore performed where combinations of IAA or 2,4-D with flavonoids were added to the nutrient solutions. From Tables 1 to 4 it is evident that most of the flavonoids to a considerable extent relieve the inhibition caused by the two auxins. The reversing effects are conspicuous also for substances such as naringenin and hesperetin, which in themselves give only weak stimulations of wheat root growth, and the stimulations observed are often greater in combinations with auxins

Table 1. *The effect of the flavanones naringenin and hesperetin (alone and in combination with some inhibitors) upon the growth of wheat roots in nutrient solution. Values in per cent of the control. Growth of the control roots 20–28 mm. (20–24 hours, 20°C). Initial root length 20–29 mm. For further details see Stenlid 1957 a.*

Flavanone added	Inhibitor, added	Conc. of inhibitor, M	Concentration of flavanone, M					
			0	10 ⁻⁶	3 · 10 ⁻⁶	10 ⁻⁵	3 · 10 ⁻⁵	10 ⁻⁴
Naringenin	—	—	100	97	100	103	102	17
"	IAA	3 · 10 ⁻⁸	32	—	39	48	68	—
"	2,4-D	3 · 10 ⁻⁷	52	—	73	92	90	—
"	"	10 ⁻⁶	34	—	49	70	74	—
"	Mannose	10 ⁻³	27	28	33	53	83	—
"	Galactose	7 · 10 ⁻⁴	40	—	—	57	—	—
"	2-DG	3 · 10 ⁻⁵	40	—	—	72	91	—
Hesperetin	—	—	100	100	104	108	74	—
"	IAA	4 · 10 ⁻⁸	46	—	53	64	—	—
"	2,4-D	4 · 10 ⁻⁷	45	—	—	75	—	—
"	Mannose	7 · 10 ⁻⁴	43	—	59	81	68	—
"	Galactose	8 · 10 ⁻⁴	43	—	60	81	64	—
"	2-DG	3 · 10 ⁻⁵	44	50	80	80	—	—

than when the flavonoids are applied alone. Some sort of antagonism or interference must therefore be supposed to exist between the flavonoids and the auxins.

Great care is necessary at the interpretation as is shown by combination experiments in which inhibitory sugars (for details about their effects see Stenlid 1957 a, b, 1959 a, b) were added together with flavonoids. Here the same type of reversal as with auxins was observed and in many cases the

Table 2. *The effect of the flavanones pinocembrin and pinobanksin upon the growth of wheat roots. For further explanations see Table 1.*

Flavanone added	Inhibitor added	Conc. of inhibitor, M	Concentration of flavanone, M					
			0	3 · 10 ⁻⁷	10 ⁻⁶	3 · 10 ⁻⁶	10 ⁻⁵	3 · 10 ⁻⁵
Pinocembrin	—	—	100	—	120	132	139	77
"	IAA	4 · 10 ⁻⁸	46	—	56	60	70	—
"	2,4-D	3 · 10 ⁻⁷	55	—	68	84	94	—
"	Mannose	7 · 10 ⁻⁴	46	—	—	79	107	72
"	Galactose	8 · 10 ⁻⁴	58	—	—	85	105	67
"	2-DG	3 · 10 ⁻⁵	41	—	—	59	—	—
Pinobanksin	—	—	100	112	119	120	128	93
"	IAA	4 · 10 ⁻⁸	45	—	51	59	71	—
"	2,4-D	4 · 10 ⁻⁷	45	—	59	76	89	—
"	"	10 ⁻⁶	30	—	—	54	76	—
"	Mannose	7 · 10 ⁻⁴	39	—	56	70	100	—
"	Galactose	8 · 10 ⁻⁴	42	—	58	74	93	—
"	2-DG	3 · 10 ⁻⁵	40	—	—	61	105	—

Table 3. *The effect of the isoflavones genistein and biochanin A upon the growth of wheat roots. For further explanations see Table 1.*

Isoflavone added	Inhibitor added	Conc. of inhibitor, M	Concentration of isoflavone, M				
			0	10^{-6}	$3 \cdot 10^{-6}$	10^{-5}	$3 \cdot 10^{-5}$
Genistein	—	—	100	102	110	120	86
"	IAA	$4 \cdot 10^{-8}$	53	—	59	71	—
"	2,4-D	$4 \cdot 10^{-7}$	58	—	96	93	67
"	Mannose	$7 \cdot 10^{-4}$	48	56	70	90	—
"	Galactose	$8 \cdot 10^{-4}$	47	64	71	81	—
Biochanin A	—	—	100	99	95	93	16
"	IAA	$4 \cdot 10^{-8}$	50	54	54	58	—
"	2,4-D	$4 \cdot 10^{-7}$	55	71	78	74	—
"	Mannose	$7 \cdot 10^{-4}$	43	74	85	88	—
"	Galactose	$8 \cdot 10^{-4}$	51	62	77	88	—

Table 4. *The effect of some flavones upon the growth of wheat roots. For further explanations see Table 1.*

Flavone added	Inhibitor added	Conc. of inhibitor, M	Concentration of flavone, M					
			0	$3 \cdot 10^{-7}$	10^{-6}	$3 \cdot 10^{-6}$	10^{-5}	$3 \cdot 10^{-5}$
Chrysin	—	—	100	105	110	110	109	—
"	IAA	$4 \cdot 10^{-8}$	38	41	43	46	48	—
"	2,4-D	$5 \cdot 10^{-7}$	39	—	59	69	61	—
"	Mannose	$7 \cdot 10^{-4}$	41	—	57	84	79	—
"	Galactose	$8 \cdot 10^{-4}$	36	—	50	63	63	—
"	2-DG	$3 \cdot 10^{-5}$	41	—	56	74	86	—
Apigenin	—	—	100	—	102	101	82	—
"	IAA	$4 \cdot 10^{-8}$	39	—	42	43	31	—
"	2,4-D	$4 \cdot 10^{-7}$	54	—	64	80	67	—
"	Mannose	$7 \cdot 10^{-4}$	41	—	51	68	53	—
"	Galactose	$8 \cdot 10^{-4}$	36	—	42	59	47	—
Quercetin	—	—	100	101	97	93	92	87
"	IAA	$4 \cdot 10^{-8}$	45	—	—	40	34	23
"	2,4-D	$4 \cdot 10^{-7}$	45	—	—	45	45	—
"	Mannose	$7 \cdot 10^{-4}$	44	—	—	44	40	41
"	2-DG	$3 \cdot 10^{-5}$	41	—	—	46	55	65
Morin	IAA	$3 \cdot 10^{-8}$	44	—	41	38	29	33
"	2,4-D	$4 \cdot 10^{-7}$	54	—	—	68	75	72
"	Mannose	$7 \cdot 10^{-4}$	33	—	—	36	36	53
"	Galactose	$8 \cdot 10^{-4}$	46	—	—	49	43	44
"	2-DG	$3 \cdot 10^{-5}$	43	—	—	55	62	88

Table 5. *The effect of combinations of naringenin and 1-NMSP upon the growth of wheat roots. For further explanations see Table 1.*

Conc. of 1-NMSP, M	Concentration of naringenin, M			
	0	10^{-5}	$2 \cdot 10^{-5}$	$3 \cdot 10^{-5}$
0	100	111	110	112
10^{-5}	151	140	125	117

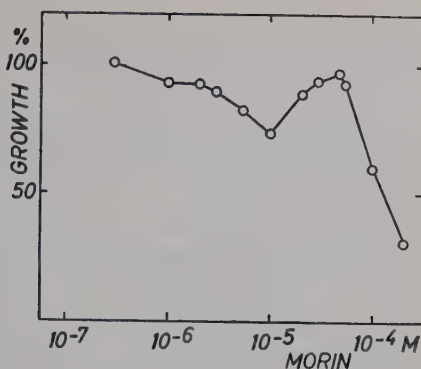


Figure 1. Effect of morin upon the growth of wheat roots in nutrient solution.

effects were even more pronounced (Tables 1 to 4). The antagonistic effect is thus not confined to auxins and not specifically anti-auxinic.

Most of the flavonoids give effects which agree in the main features, although the quantitative response of the roots varies a great deal. Morin, however, behaves in a diverging way (see Figure 1 and Table 4). In rather low concentrations the growth is distinctly inhibited, whereas somewhat higher concentrations are less inhibitory. This results in a complicated concentration-growth response curve. The inhibition caused by IAA is enhanced by low concentrations of morin; the inhibition caused by 2,4-D is reversed to some extent. Morin gives a distinct reversal of the inhibition caused by 2-deoxyglucose, a much less pronounced reversal of that caused by mannose and no distinct effect against galactose. Quercetin, which is structurally near related to morin, is also rather ineffective as an antagonist (Table 4). Di Maggio (1952) reported an auxin-like stimulating activity of quercetin upon the germination of wheat caryopses. Gowing and Leeper (1961) who tested quercetin in the pea test did not find any conspicuous effects.

The effects of morin remind of those exerted by triiodobenzoic acid and by (+)2-isopropyl-4-chloro-5-methylphenoxy- α -propionic acid (see Åberg 1961). Their effects have been interpreted as composed of a synergism with IAA at low concentrations, an effect of anti-auxinic type at higher concentrations, and finally a nonspecific toxicity at still higher concentrations.

The flavonoids behave similar to the antiauxins with respect to their effects upon root hairs. Antiauxins, when applied in concentrations causing an increased root elongation, at the same time inhibit the formation and growth of root hairs (personal communication from unpublished experiments by Dr. Ivar Ekdahl, who has found this effect for various antiauxins; cf. Hansen 1954 p. 235). Such effects also were obtained with the substances which gave antagonistic effects against auxins in this investigation.

At concentrations of flavonoids, which are effective as antagonists against auxins or which give stimulations of root growth in pure solutions, the formation and length of root hairs is considerably decreased at those parts of the roots which have grown out in the test solution applied. The substances with weak effects against IAA (morin, quercetin and apigenin) do not affect root hair growth very much.

Hendershott & Walker (1959 b) found naringenin inhibitory to the growth of wheat coleoptiles. The concentrations applied were rather high, and it is not clear if the effect was due to antagonism against auxin or to an un-specific inhibition.

The growth stimulations caused by the antiauxinic substance 1-NMSP are distinctly reversed through addition of naringenin (Table 5). Naringenin is thus active against both auxins and antiauxins. A similar activity has been reported for *i.a.* 1,3-dichloro-2-naphthoxyacetic acid (Hansen *et al.* 1955).

The uptake of chloride, nitrate, and phosphate ions is inhibited by the flavonoid pigments (Table 6). As a rule distinct inhibitions are obtained at concentrations which give positive effects upon root growth. The uptake of sugars is inhibited only by concentrations higher than those necessary to inhibit ion absorption (Table 8).

Attempts to reverse the inhibitory effects of mannose upon chloride absorption through addition of flavonoids were not successful (Table 7) and it

Table 6. *The effect of some flavonoid substances upon the uptake of chloride, nitrate, and phosphate ions in excised wheat roots.* The values give the absorption in per cent of the control without flavonoids. Ion absorption determined through analyses of the external solution at the beginning of the experiments and after 4 hours. For further details see Stenlid (1957 b and 1959 a).

Flavonoid substance added	Ion absorbed	Concentration of flavonoid, M				
		10 ⁻⁶	3 · 10 ⁻⁶	10 ⁻⁵	3 · 10 ⁻⁵	10 ⁻⁴
Naringenin	chloride	99	96	88	69	31
"	nitrate	103	90	70	34	11
"	phosphate	83	76	78	46	—
Hesperetin	chloride	102	94	91	79	—
"	nitrate	97	95	85	69	—
Pinobanksin	chloride	95	91	61	33	—
"	nitrate	103	100	70	22	—
Pinocembrin	chloride	99	91	77	56	—
"	nitrate	97	84	46	25	—
Genistein	chloride	100	99	85	80	—
"	nitrate	102	90	77	39	—
Biochanin A	chloride	97	91	74	34	—
Morin	"	—	86	83	74	51
"	nitrate	108	106	97	76	—
Quercetin	chloride	107	112	108	108	—
"	nitrate	101	97	91	78	—

Table 7. *The effect of mannose in combination with flavonoids and with dinitrophenol upon the absorption of chloride ions by excised wheat roots. The values give the absorption in μg ions/4 hours and 100 mg. dry matter.*

Abs. of control	Conc. of mannose, M	Reversing substance added	Conc. of reversing substance, M				
			0	10^{-6}	$3 \cdot 10^{-6}$	10^{-5}	$3 \cdot 10^{-5}$
13.3	$7 \cdot 10^{-4}$	Quercetin	6.1	—	—	—	6.5
13.3	$7 \cdot 10^{-4}$	DNP	6.1	5.6	—	—	—
13.3	$7 \cdot 10^{-4}$	Morin	6.1	—	—	5.5	—
13.1	$7 \cdot 10^{-4}$	Naringenin	5.8	—	6.0	5.6	—
13.1	$7 \cdot 10^{-4}$	Genistein	5.8	—	—	6.1	—
13.4	10^{-3}	DNP	4.1	—	3.5	3.2	—
10.8	10^{-3}	DNP	3.8	—	3.6	—	—
14.4	10^{-3}	Hesperetin	3.6	3.7	3.8	2.9	—
10.8	10^{-3}	Naringenin	3.8	—	3.7	—	—

Table 8. *The effect of some flavonoids upon the absorption of sugars by excised wheat roots. The experiments were made in 0.003 M phosphate buffer containing CaCl_2 ($5 \cdot 10^{-4} M$) and sugar (0.002 M). The values give the absorption in per cent of the control without flavonoid. Sugar determinations were made on samples taken from the external solution at the start and after 4 hours (cf. Stenlid 1957 a).*

pH of nutrient solution	Sugar added	Flavonoid added	Conc. of flavonoid, M				
			$3 \cdot 10^{-6}$	$2 \cdot 10^{-5}$	$3 \cdot 10^{-5}$	$6 \cdot 10^{-5}$	10^{-4}
7.0	Glucose	Naringenin	—	—	102	89	64
6.0	"	"	—	99	—	79	20
6.0	Galactose	"	—	104	—	61	—
7.0	Glucose	Biochanin A	97	—	—	—	—
6.0	"	"	92	—	—	—	—
7.0	"	Hesperetin	—	—	109	—	—
7.0	"	Pinobanksin	—	—	88	—	—

seems as if the flavonoid pigments have no restoring effect at all against the mannose inhibition of chloride absorption. Nor does dinitrophenol reverse this inhibition (Table 7, cf. Stenlid 1959 a, Figure 2). The antagonistic effects of flavonoids seem thus to be specific for growth. Sugars (*e.g.* glucose) reversing the inhibitions caused by mannose are effective in both the growth and ion absorption processes (see Stenlid 1957 a).

Oxygen uptake in the roots is not altered very much after the addition of naringenin, quercetin or pinocembrin (unpublished experiments, which will be reported in other connection).

Discussion

The effects of the flavonoid pigments are similar both to those obtained with substances of anti-auxinic nature and to those exerted by the uncoupling agent dinitrophenol. Also dinitrophenol counteracts the growth inhibi-

tions caused by auxins and sugars and may in itself give slight stimulations of root growth (Stenlid 1959 a). An interpretation which is close at hand is that both dinitrophenol and the flavonoids decrease the uptake and transport of the inhibitory substances through uncoupling effects upon oxidative phosphorylation. This would give a simple explanation of the reversing effects against different types of substances (sugars, auxins, antiauxins). Also the inhibition of ion absorption might be explained as due to an effect upon oxidative phosphorylation leading to a decreased generation of adenosine-triphosphate.

Some facts, however, are not easy to explain with this simple view. Concentrations of naringenin, which alleviate the galactose and mannose inhibition of growth conspicuously, do not affect the absorption of glucose and galactose to any considerable degree. Nor is there any reversal of the inhibition of chloride absorption caused by mannose. These results do not speak in favour of a hypothesis that the antagonistic effects of the flavonoids against the sugars should be due to a general inhibition of sugar absorption. No stimulations of oxygen uptake or of chloride absorption comparable to those caused by dinitrophenol have been obtained with the flavonoids. Other uncouplers deviate from dinitrophenol in these respects, however (unpublished experiments by the author, cf. data for methylene blue in Stenlid 1950), and these results do therefore not exclude the possibility that the flavonoids act as uncoupling agents.

Another complication is that no complete parallelism is observed between the effects upon the endogenous growth mechanism and upon the action of externally applied substances. If in addition the deviating effects of morin are kept in mind, it is clear that a single mechanism at one site cannot be responsible for the effects observed.

In view of the obvious similarities between the activity of flavonoid substances and dinitrophenol it is nevertheless tempting to ascribe to the flavonoids an uncoupling effect upon oxidative phosphorylation. A further support for such a hypothesis is that other well-known uncoupling agents (*e.g.* dicoumarol, usnic acid and methylene blue) act in a similar way as the flavonoids (unpublished experiments by the author). Direct tests of the effects upon oxidative phosphorylation in mitochondria are necessary to decide to what extent the effects caused by the flavonoid substances are due to an interference with oxidative phosphorylation, however. A detailed discussion of this question and of the possible connection between anti-auxinic and uncoupling properties must therefore be deferred until later. It is quite possible that the flavonoids have two different activities influencing growth: one uncoupling leading to antagonistic effects on externally applied substances and one effect of other kind ("antiauxinic") on the endo-

genous growth mechanism. For a discussion of the stimulations of root growth caused by externally applied substances see *e.g.* Torrey (1956) and Åberg (1957). A support for a hypothesis that the flavonoids act in the same way as some synthetic antiauxins is that the stimulation of root growth caused by 1-NMSP is counteracted by naringenin (see Table 5) indicating competition at a common site of action.

It is in any case impossible to explain all the results obtained with combinations of inhibitors and flavonoids as due to additive effects as even flavonoid concentrations which are inhibitory in themselves may give conspicuous reversal of inhibitions. Other results which are not explainable as additive effects are that substances without stimulating effects in themselves give reversal and that the antagonistic effect against 2,4-D is greater than that against IAA.

The complications mentioned are of the same kind as those encountered in the interpretation of the effects obtained with synthetic antiauxins. Quite as for synthetic antiauxins species differences are to be expected, and some preliminary experiments have shown that the effects of flavonoids upon flax roots deviate from those obtained on wheat roots.

The number of substances tested is rather limited compared to the large number of naturally occurring flavonoids. It is at present impossible to draw any conclusions as to the specific effects of substitution with hydroxyl groups at special positions etc., especially as different pK values and ionization of the substances certainly influence the absorption of them. Of the substances tested the flavone apigenin, the isoflavone genistein and the flavanone naringenin all have their three hydroxyl groups in the positions 5,7 and 4'. They are all active (apigenin only very slightly against indole acetic acid, however) and judging from the present material all the three groups of flavonoids tested seem to interfere with root growth at low concentrations. The only general feature possible to discern is that all the four flavones tested are rather inactive as antagonists against indole acetic acid and that they do not give any pronounced stimulations of root growth in themselves.

The results with combinations of mannose and flavonoids show that it is possible to distinguish between the inhibitory effects of mannose upon growth and upon chloride absorption. It is true that the concentrations of flavonoids applied in the experiments in themselves are slightly inhibitory to chloride absorption but nevertheless an interaction of the same kind as that observed in the growth seems improbable. The same lack of interaction in chloride absorption is found for the combination dinitrophenol-mannose. Here the concentration of dinitrophenol (reversing the growth inhibition of mannose) in itself even slightly stimulates chloride absorption (see Stenlid 1959 a, Figure 2). The conclusion must therefore be that the inhibitory effects

of mannose upon chloride absorption and growth are manifested at different places in the reaction chain of the sugars or that the two effects are coupled to different carbohydrate pathways. These results confirm the earlier expressed view (see Stenlid 1957 a, 1959 b) that the inhibitions caused by sugars are rather complex. Not only do the various sugars differ in their effects, but it seems as if the action of one sugar (*e.g.* mannose) is not explainable by a single mechanism.

Flavonoids, especially quercetin and eriodictyol, are known to stimulate the enzymatic oxidation of ascorbic acid (see Huszák 1937, Neuman *et al.* 1960), and different phenols (including dinitrophenol) enhance the activity of indole acetic acid oxidase (see Goldacre *et al.* 1953, Pilet 1961). The possible participation of flavonoids in such reactions may indirectly influence other processes, but with the available experimental material speculations in this direction cannot explain the effects of the flavonoids.

Many flavonoids form complexes with metals, *e.g.* copper and this can also influence enzymatic reactions, although in an inhibitory way (see Heiman and Heinrich 1960, Crawford *et al.* 1961).

No definite hypothesis can at present be given for the mode of action of the flavonoid pigments, and their possible importance in the living plant cannot yet be assessed. This is not surprising as the mechanism of root growth and the role of auxins in it is still incompletely known. Further investigations are desirable as commonly occurring flavonoids in low concentrations have distinct effects on important processes in plant roots. It must be remembered, however, that the largest portion of the flavonoids is present as glycosides in the vacuole and that the influence of substances in the vacuole pool upon processes in the cytoplasm is rather limited.

Summary

Some flavonoid substances (flavones, isoflavones, and flavanones) were tested for their effects upon wheat seedling roots. In concentrations of 10^{-6} to 10^{-5} M most of the flavonoids enhanced root growth and relieved the inhibition caused by externally added indole acetic acid and 2,4-D. Also growth inhibitions caused by inhibitory sugars were reversed. The flavones did not relieve inhibitions caused by indole acetic acid to the same degree as did the other substances. Morin gave complicated growth effects of the same type as those reported for triiodobenzoic acid.

The absorption of chloride, nitrate and phosphate, but not that of glucose was inhibited by flavonoids at concentrations stimulating growth. No distinct effects upon oxygen uptake were obtained.

The effects of the flavonoids in certain respects resemble those caused by antiauxins and by agents uncoupling oxidative phosphorylation.

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Galactose as a Possible Source of Carbon for the Growth of Isolated Roots of Cucumber

By

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In a previous communication (Stenlid 1959) it was reported that galactose did not inhibit the growth of seedling roots of intact young plants of *Cucumis sativus* and *Lepidium sativum*. Moreover, galactose counteracted inhibitory effects caused by some other sugars upon these roots. Nine other plant species were tested, and for all of them it was found that root growth was inhibited by galactose under similar conditions. From these experiments it could not be concluded whether galactose was really utilized for the growth by cress and cucumber roots or if it was only non-inhibitory and indifferent. In the present paper some experiments are reported indicating that isolated roots of cucumber in contrast to cress roots are able to utilize galactose to some extent as a source of carbon for root growth.

Methods

The following species have been used in the experiments: *Cucumis sativus* (Weibull's Favör), *Cucumis melo* (Weibull's Billeberga), *Cucurbita pepo* (matpumpa, Vegetable Marrow), *Lepidium sativum* (Weibull's trädgårdskrasse), *Isatis tinctoria* (see Danckwardt-Lillieström 1957), *Brassica napus*, var. *napobrassica* (Svalöf's Viktoria), *Sinapis alba* (Svalöf's Primex), and *Helianthus annuus*. Growth experiments were performed with intact seedlings (all of the species mentioned above) and with isolated roots (*Cucumis sativus*, *C. melo*, *Lepidium sativum*, and *Isatis tinctoria*). The growth experiments with intact seedlings were performed as described by Stenlid (1959). For the experiments with isolated roots seeds were sterilized with hypo-

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chlorite and placed on moist filter paper in Petri dishes at 25°C. Root tips from the main root with a length of 10 mm. were transferred to 100 ml. Erlenmeyer flasks containing 50 ml. of the nutrient solution described by Bonner (1940) as modified by Danckwardt-Lillieström (1957). Iron was added as FeNa-EDTA. In one experiment with cucumber tips from lateral roots were used. The culture flasks were kept at 25°C. in darkness. Two root tips were placed in each flask and at least five flasks were used for each sugar concentration applied.

Experimental Results

Isolated roots of cucumber were found to grow on glucose as well as on sucrose and galactose (Table 1). The growth ceased after some time, however, and only a limited growth was thus obtained in the solutions applied. A rather great variation between individual roots was observed with all these sugars but none of them was found conspicuously superior to the others. Some roots in 2 % galactose increased in length to over 100 mm. Unfortunately isolated roots of species belonging to Cucurbitaceae are notoriously difficult to cultivate (Robbins 1951) and it is therefore not surprising that the results with cucumber roots are rather irregular. Lateral roots were easily formed from the isolated main root tips, which shows that cell division and elongation takes place also in the primordia growing out in the galactose solution. It is not possible that the native supply of sugars is sufficient for the growth observed, and it can be concluded that galactose is utilized for growth to about the same extent as glucose and sucrose. Attempts to subcultivate root tips or lateral roots formed from the isolated roots were not successful. Lateral roots taken from the seedling roots in the Petri dishes gave a restricted but rather regular growth (Table 1). Obviously some factor necessary for unlimited growth is lacking in the nutrient solution applied. With the inhibitory sugar mannose cucumber roots showed no growth at all.

Isolated roots of *Lepidium sativum* did not grow on galactose although roots of intact seedlings of cucumber and cress behaved in the same way (see Stenlid 1959).

Burström reported that the growth of sunflower and flax roots was not inhibited by galactose (1948). Farkas (1954) and Ferguson *et al.* (1958) found galactose inhibitory to these species, however, and also with the methods used in this investigation both flax roots (see Stenlid 1959) and sunflower roots (Table 2) of intact seedlings were distinctly inhibited by galactose.

To investigate if resistance to galactose is possibly a property specific to certain families some additional species belonging to Cucurbitaceae and Cruciferae were tested (see Table 2). Roots of intact seedlings of *Cucurbita*

Table 1. *Growth of isolated roots in nutrient solution with different sugars as a source of carbon. The values give the root length and number of lateral roots at the end of the experiments. As a rule the values are the mean of at least 10 roots (in a few cases less, as some roots had to be excluded due to infection).*

Plant species	Duration of experiments, days	Sugar added	Concentration of sugar %					
			0.5		1.0		2.0	
			Root length mm.	Numb. of laterals	Root length mm.	Numb. of laterals	Root length mm.	Numb. of laterals
<i>Cucumis sativus</i>	11	Glucose	28	4	34	7	41	16
".....	11	Galactose	26	11	34	15	51	16
".....	11	Sucrose	39	10	48	7	—	—
" (laterals).....	8	Glucose	—	—	—	—	23	5
" ".....	8	Galactose	—	—	—	—	25	5
" ".....	8	Sucrose	—	—	—	—	22	4
<i>Lepidium sativum</i>	7	Glucose	—	—	—	—	40	0.3
".....	7	Galactose	—	—	—	—	13	0
".....	7	Sucrose	—	—	—	—	45	2
<i>Isatis tinctoria</i>	21	Glucose	22	0	23	0.6	18	0.7
".....	21	Galactose	10	0	10	0	10	0
".....	21	Sucrose	58	4	81	6	82	5

Table 2. *The effect of galactose upon the growth of roots of intact seedlings in nutrient solution (24 hours, 20°C). Values in per cent of growth in control solution without sugar.*

Plant species	Conc. of galactose <i>M</i>	
	$3 \cdot 10^{-3}$	10^{-2}
<i>Lepidium sativum</i>	122	140
<i>Isatis tinctoria</i>	38	—
<i>Sinapis alba</i>	63	—
<i>Brassica napus</i> v. <i>napobrassica</i>	18	—
<i>Cucumis sativus</i>	108	110
— <i>melo</i>	98	103
<i>Cucurbita pepo</i>	43	—
<i>Helianthus annuus</i>	22	—

pepo were inhibited by galactose whereas those of *Cucumis melo* were not. Isolated roots of *Cucumis melo* showed a very slow growth on glucose, galactose and sucrose and nothing can be said about their ability to utilize galactose. The root growth of seedling roots from three cruciferous species was inhibited by galactose. Ability to endure or utilize galactose does thus not seem to be a common property in the families Cucurbitaceae and Cruciferae.

In the roots studied three different modes of reaction towards galactose have thus been observed:

- 1) The growth of intact seedling roots (and most probably also of isolated roots) is inhibited by galactose. Most species seem to react in this way.
- 2) The growth of intact seedling roots is not inhibited by galactose; isolated roots cannot utilize galactose for growth (*Lepidium sativum*).
- 3) The growth of intact seedling roots is not inhibited by galactose; isolated roots are able to utilize galactose for growth (*Cucumis sativus*).

Several cases are known where plant tissues may utilize galactose for growth (see Gautheret 1945, Hildebrandt and Riker 1949, Ball 1955). As far as we know, however, cucumber roots is the first case where an actively growing isolated root has been found to utilize galactose for growth.

Summary

Isolated roots of *Cucumis sativus* are able to utilize galactose for growth to some extent. Unlimited growth was not possible to obtain with this species and the variation between individual roots was great. Some of the roots

showed a rather good but limited growth, and galactose seems to be about equivalent to glucose as a source of carbon for cucumber roots.

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The Effect of Gibberellic Acid on the Inhibitor β Complex in Resting Potato

By

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It is well documented (Hemberg 1947, 1949, 1952, 1954, 1958 a; Blommaert 1954; Varga and Ferenczy 1957) that the peel of resting potato tubers contains acid, ether-soluble substances with growth-inhibiting action and that these substances towards the end of the rest period decrease in amount and tend to disappear completely. This inhibitor complex, called inhibitor β , disappears also when the rest is broken off artificially with ethylenechlorhydrin or glutathione (Hemberg 1949, 1950; Varga and Ferenczy 1956, 1957; Szalai 1958).

A few years ago it was reported (Brian, Hemming and Radley 1955; Rappaport, Lippert and Timm 1957) that the treatment of resting potato tubers with gibberellic acid (GA) breaks their rest. Since the results from numerous investigations indicate that the gibberellins are a group of natural plant hormones, it should be of special interest to attempt to investigate the mechanism of this rest-breaking effect. The aim of the present study has been to determine whether a treatment of resting potato tubers with GA affects their content of inhibitor β .

Material and Method

The material employed in this investigation was potato of the variety Majestic, which is known to have a very long rest period. The potatoes were obtained from the Institute for Plant research and Cold Storage in Nynäshamn, where they had been stored in the dark at a temperature of +4°C. The work was started on December 2, 1960.

In the treatment with GA the potato tubers were placed in a solution containing 50 mg. of GA per liter of distilled water. The acid was first dissolved in a few drops of ethanol in order to facilitate its dissolution in water. In parallel experiments potato tubers were treated with distilled water without GA but with the same amount of ethanol. As is seen in Figures 1 and 2 two treatment periods of different duration were employed. In the first case the potato tubers were treated 2×2 hours. This treatment was carried out during the course of 72 hours. In the second case the treatment was extended to 2×12 hours during the course of 72 hours. After the treatment the potato tubers were placed in a ventilated dark room on moist sand at $+20^{\circ}\text{C}$. The sampling took place 3, 6, 10, and 17 or 32 days after the onset of the treatment. Each test comprised twelve tubers. Ether extraction of the peel was performed according to Hemberg's (1952) method and the extract was separated into acid and neutral fractions according to Larsen's (1955) modification of Boysen Jensen's (1941) method II.

The acid fraction was chromatographed (ascending chromatography) in *n*-butanol — ammonia (sp. gr. 0.91) — water (100 : 4.2 : 16.8) on Whatman no. 1 paper. In this system, according to Blommaert (1954), inhibitor β has the R_f value of 0.52–0.74, well separated from indole-3-acetic acid with the R_f value of 0.28–0.30 and also from GA, which in this system has the R_f value of 0.24 according to Simpson (1958). The chromatograms were run in tubes, one chromatogram in each, according to Nitsch (1956). Each chromatographing paper was 2.5 cm wide and was allowed to hang in the chromatographing solvent until the front had risen 15 cm. above the spot of application. The chromatograms were dried thereafter in a stream of air and tested biologically with the *Avena* straight growth test. The test was carried out according to Hemberg's (1958 b) method with oats of the variety Brighton as the experimental material.

Results and Discussion

The results of the gibberellin treatment 2×2 hours are shown in the form of histograms in Figure 1. From these it is seen that the gibberellin treatment affected the inhibitor β content of the resting potato tubers. The amount of inhibitor decreased. The effect was noticeable 3 to 6 days after the onset of the treatment, after 10 days fully evident. In the untreated tubers, on the other hand, the amount of inhibitor β was fairly constant from test to test.

In Figure 2 are presented the results of the longer gibberellin treatment (2×12 hours). Also here the amount of inhibitor β in the treated tubers decreased, whereas that in the untreated ones was practically unchanged throughout the series, although with a certain decline after 32 days. Any certain difference in effect between the shorter and the longer gibberellin treatment has not been demonstrated.

Various theories have been proposed regarding the physiological causes of the rest of potato. The fact that the inhibitor β content decreases towards the end of the resting period has given rise to the hypothesis that this inhibitor complex is the factor that controls the continuance of the rest. For different

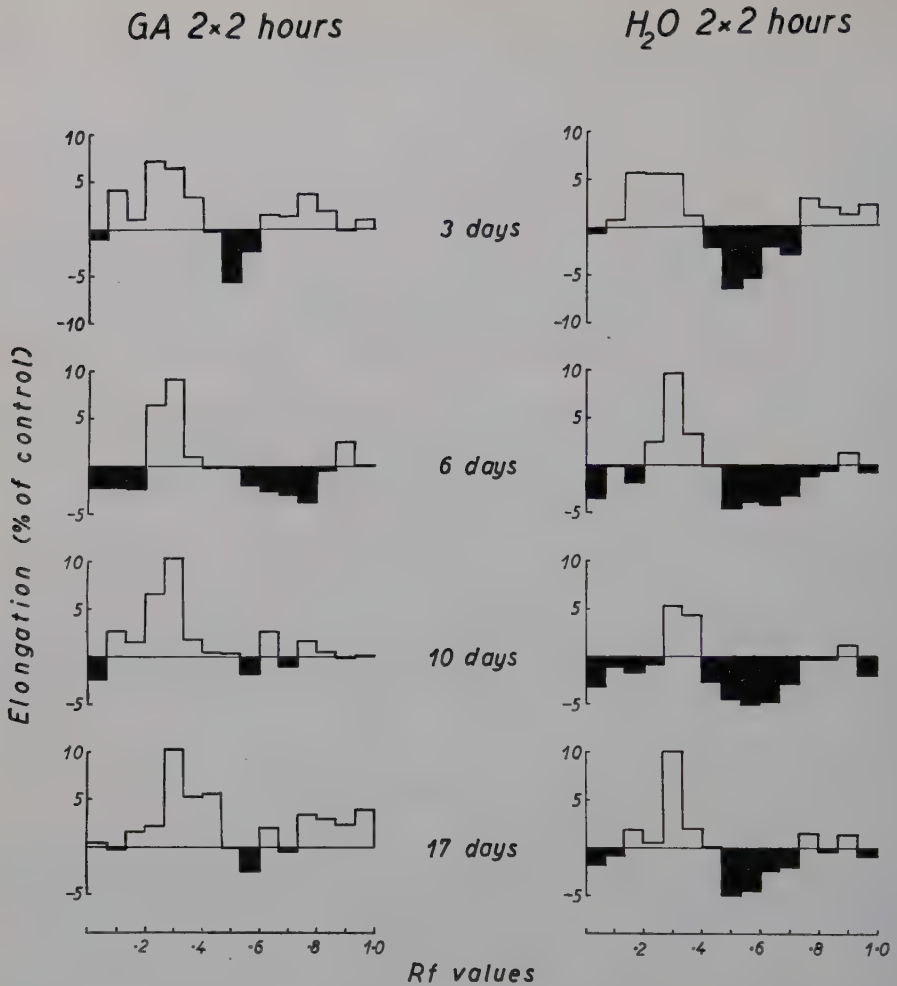


Figure 1. *Biologic determination of chromatograms of the acid fraction of ether extracts of potato peel.* To the left: from tubers treated with an aqueous solution of gibberellic acid (GA) 2×2 hours during the course of 72 hours. To the right: from untreated samples, i.e., treated only with water. Extract from 2.5 g. of potato peel is transferred to each chromatogram. Number of days denotes the time from the onset of the treatment to the sampling.

reasons doubts have been raised against this hypothesis (Burton 1956; Housley and Taylor 1958; Buch and Smith 1959). Certainly the regulation of the rest is a complicated process. However, the results of the present investigation combined with the inhibitor hypothesis give *one* explanation as to why gibberellin

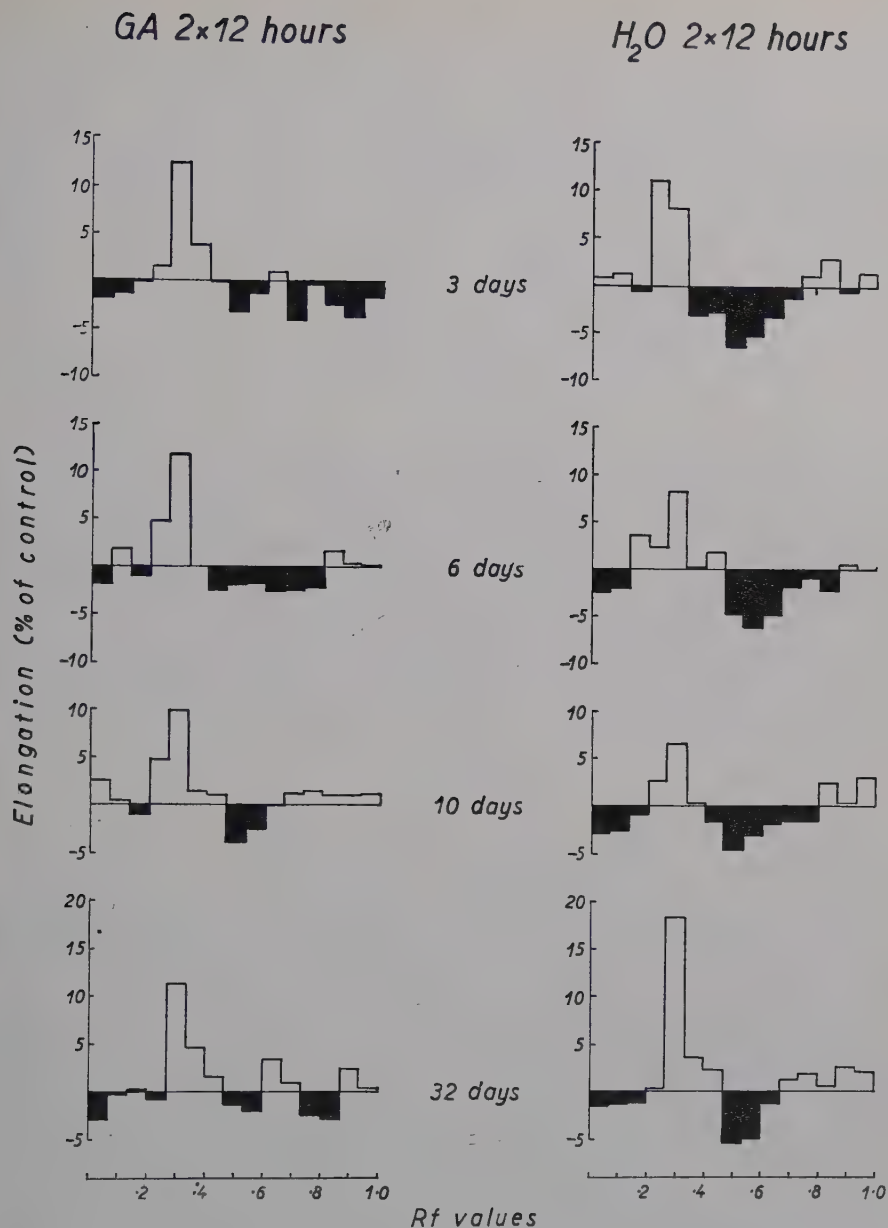


Figure 2. Biologic determination of chromatograms of the acid fraction of ether extracts of potato peel. To the left: from tubers treated with an aqueous solution of gibberellic acid (GA) 2 × 12 hours during the course of 72 hours. To the right; from untreated samples. i.e. treated only with water. Otherwise as in Figure 1.

treatment is capable of breaking the rest of potato tubers. Furthermore, the experimental results justify the question of whether endogenous gibberellins play a role in the natural regulation of the resting period of potato. Endogenous gibberellins have been demonstrated in potato tubers as well as in growing potato plants (Okazawa 1959). A pronounced increase in the content of endogenous gibberellins of potato tubers in the transition from rest to sprouting has recently been reported (Smith and Rappaport 1961). However, this report does not clarify whether the increase of the gibberellin content precedes the sprouting or vice versa.

Summary

The treatment of resting potato tubers with an aqueous solution of gibberellic acid reduces the inhibitor β content of the tubers, which has been demonstrated by means of the *Avena* straight growth test on chromatograms from purified ether extracts. The effect was noticeable after 3 to 6 days and fully evident 10 days after the treatment. No differences in effect from treatment periods of different duration could be demonstrated.

I wish to thank Professor Torsten Hemberg for valuable advice and generous help during the performance of this investigation.

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**Inhibition of Growth and Panicle Formation in Oats
Induced with an Extract of the Leafhopper
Calligypona pellucida (F.)**

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Plant diseases caused by the salivary toxins of phytophagous insects with sucking mouth-parts occur in all parts of the world and cause severe losses in the yield of several different crop plants. Very few facts are known, however, about the chemical nature of plant diseases of this type. 3-Indoleacetic acid and amino acids have been detected in the salivary secretions of Homoptera and Heteroptera and have been suspected to be the active compounds, especially in cases where gall formation is a symptom of plant injury but also, although to a lesser extent, in cases where plant growth inhibition occurs (Link *et al.* 1940; Nystrakakis 1946, 1947, 1948 a-c; Allen 1947, 1951; Kloft 1950, 1960 a-b; Balch 1952; Duspiva 1954; Nuorteva 1956 a; Anders 1957, 1958 a-b; Kloft and Ehrhard 1959; Schwarzbach 1959; Schällér 1959, 1960; Nuorteva and Laurema 1961). It has been possible to simulate the phytopathogenic effects of given leafhopper species by injecting autoclaved extracts of these insects (Martin 1942), using certain amino acids (Anders 1957, 1958 a-b) or 3-indoleacetic acid (Balch 1952). Several hydrolyzing

enzymes (amylase, maltase, invertase, α -galactosidase, β -glucosidase, lipase, esterase, different proteinases, phosphatase, phosphorylase and polygalacturonase) have also been detected in the salivary secretions of different phytophagous Homoptera and Heteroptera (Baptist 1941; Kretovich *et al.* 1943 a-b; Kretovich 1944; Goodchild 1952; Duspiva 1953, 1954; Saxena 1954 a-c, 1955; Nuorteva 1954 a-b, 1955, 1956 b-c, 1958 a-b; McAllan and Cameron 1956; Adams and McAllan 1956, 1958; Saxena and Bhatnagar 1958; Miles 1960 a; Laurema and Nuorteva 1961). Different plant diseases have been suspected to result from the action of these enzymes but there is very little real evidence regarding their activity in the living plant. Nor is much known about the significance of the various other salivary substances (*e.g.* lipo- and mucoproteins and oxidase; see Miles 1959, 1960 a-b) as plant pathogens.

During recent years the causes underlying the phytopathogenicity of the auchenorrhynchous homopteran *Calligypona pellucida* (F.) (Araeopidae) have been intensively studied on account of the great damage to oats caused by this leafhopper in some areas of Sweden (on the literature see Lindsten 1960 a), Czechoslovakia (Dlabola 1957; Pruša 1958; Pruša *et al.* 1959), Finland (Kanervo *et al.* 1957; Raatikainen and Tinnilä 1957), and England (Slykhuis and Watson 1958; Watson 1959). Although widespread and abundant, this species damages oats only in restricted areas — in Finland over a comparatively extensive western coastal area. It has been stated that *Calligypona pellucida* causes the damage by transmitting two virus diseases, oat sterile-dwarf and European striate mosaic (Pruša 1958; Slykhuis and Watson 1958; Pruša *et al.* 1959, 1960 a-b; Lindsten 1960 a-b; Watson 1959; Watson and Sinha 1959; Vacke 1960; Ikäheimo 1960, 1961) but also by means of a nonviral plant growth inhibiting salivary toxin (Nuorteva 1958 c, 1959). The occurrence of the oat sterile-dwarf virus and of the nonviral salivary toxin seems to be confined to the areas where *Calligypona pellucida* causes damage to oats. Examinations of dissected salivary glands have shown that proteases, amylases, and polygalacturonase are absent from the salivary glands of *Calligypona pellucida* specimens of both injurious and uninjurious populations (Nuorteva 1958 c; Laurema and Nuorteva 1961).

The authors have embarked on a study of the chemical nature of the salivary secretions of *Calligypona pellucida*. The purpose of the present paper is to report preliminary results obtained by injection of an extract of these leafhoppers into oat plants and attempts to isolate the active substance. Some negative results concerning the occurrence of amino acids and 3-indoleacetic acid in the dissected salivary glands are also reported.

Experiments on the Effect of Leafhopper Extracts on Oats

The leafhopper material for the preparation of extracts for the injection experiments was collected by Mr. Teuvo Suominen and Mr. Raimo Hissa in the period 6.-17.7.1958 from the commune of Mustasaari, which is situated in the area of oat damage. The leafhoppers were collected from oat fields with hoop nets. After slight narcosis with ether the leafhoppers were preserved in 70 per cent ethanol. The total catch amounted to about 85,000 specimens weighing about 85 g. During the summer of 1959 and 1960 two similar catches of leafhopper material were collected in the same area by Mr. Matti Kivinen and collaborators. As reported below, the phytopathogenic effects of extracts obtained from the first material was already relatively weak and from the latter they could hardly be proved. It is of interest to note that it was during years after the sudden disappearance of the oat damage in Finland that the ineffective catches were collected, whereas the effective catch was collected during a year in which damage occurred in the oat fields from which the material was taken.

The extraction procedure was continued in the laboratory as follows: The ethanol solution was filtered off from the leafhoppers, which were washed once more with 70 per cent ethanol. The total amount of solution was then 1,700 ml. The ethanol was distilled off, about 400 ml. of aqueous extract remaining. In this extract a small amount of reddish precipitate settled to the bottom, the bulk of the extracted material remaining as a light brown suspension in the solution. It could not be separated by filtering or centrifuging. The suspension was mechanically homogenized twice, and centrifuged in between. The final suspension was very finely and evenly divided, no solid substances precipitating from it. The water suspension thus obtained was used in all experiments. It was calculated that 1 ml. of suspension corresponded to about 200 mg. of whole leafhoppers. Guldregn II oats (commercial grain) were used in the pot experiments with quartz sand as substrate.

Experiment 1 (16.7.-9.10.1958). Each pot contained 11 oat plants. The oats were sown on 16.7., and the leafhopper extracts was injected on 19.8. when the plants were about 30 cm. high. The stem then had 3 to 4 nodes. The injection was performed with injection needle No. 18, so much liquid being forced into the stem in four places at different levels that the surplus dribbled away from the axil of the leaf when the hollow of the stem was filled. The amount injected was 1 ml. per stem, about 0.6 ml. of which was calculated to have run away. Hence about 0.4 ml. of leafhopper extract would have remained in the plant. This is a rough estimate, and the amount of liquid remaining in different individual plants varied somewhat. The number of oats injected with leafhopper extract was 11. These plants are referred to below as LH-injected oats.

1 ml. of water was injected into the oats in the other pot in the same way. The number of oat plants injected with water was also 11 (water-injected oats). A third pot with 10 plants into which nothing was injected served as a control.

Nothing exceptional, barring a slight bending of the stems, was noted in the test plants (LH-injected and water-injected oats) immediately after the injection. The wounds seemed to heal rapidly; in the LH-injected oats they turned brown in a short time.

A week after the injection, the LH-injected oats were observed to lag behind the controls in regard to growth in height. In the water-injected oats growth was also retarded somewhat. The effect was much smaller, however, than in LH-injected oats. On 26.8., the average height of the LH-injected oats was 33.1 cm. and that of the water-injected oats 38.1 cm. The measurements were made to the end of the sheath of the top leaf.

30.8. The water-injected oats began to form panicles. On the control oats the formation of panicles had advanced somewhat farther than on the water-injected oats. No panicles were to be seen on the LH-injected oats.

2.9. Ten of the water-injected oats had panicles, but none of the LH-injected oats had any. The growth of the stem between the last two nodes in the latter seemed especially to lag behind the corresponding growth of the water-injected oats and the control oats. In all other respects the LH-injected oats were vigorous and green. When the growth of the main shoot ceased, they began vigorously to form tillers. The heights of the plants were: LH-injected oats 33.5 cm., and water-injected oats 41.3 cm. The photograph taken on 4.9 clearly shows the state of growth of the test plants and the controls (Figure 1).

11.9. The LH-injected oats began to form panicles on the tillers. On the water-injected oats and control oats the flowering of the main shoots had

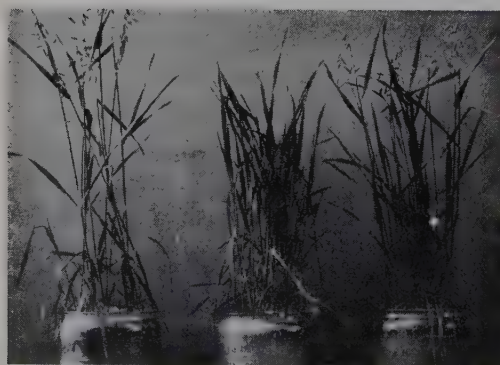


Figure 1. *The experiment with oat plants, 16.7.-9.10.1958. Left: control plants without injection. Middle: plants injected with the leafhopper extract described in the text. Right: plants injected with the same amount of water as the latter plants with extract. The photograph was taken on 4.9.1958.*



Figure 2. *The same experiment as in Fig. 1, with the exception that the photograph was taken on 21.9.1958.*

ceased: No panicles had been formed on the main shoots of a single LH-injected oat plant.

18.9. The main shoots of the LH-injected oats wilted rapidly. When the stems had wilted, they broke at the point of injection. Panicles had already been formed on the lateral shoots. The photograph taken on 21.9. gives a clear picture of the growth of the oats at this stage (Figure 2).

9.10. New panicles were no longer formed even on the tillers, and growth had ceased in all groups. The number of panicles in the different test plants during experiment 1 are given in Table 1.

Experiment 2 (9.6.–31.8.1959). All the oats were grown in the same pot. An LH-extract was injected into three of the plants, water into two, and nothing into two (controls). A No. 20 needle was used for the injections, which were begun when the plants were only 10 to 17 cm. high. Injections were now given daily, the dose per plant being small (0.1 ml.). No appreciable amount of liquid was now wasted. During the period 6.7.–14.7., 0.9 ml.

Table 1. *The number of panicles in experiment 1.*

Material	2.9.		18.9. and 9.10.	
	Main shoots	Tillers	Main shoots	Tillers
LH-injected oats	0	0	0	10
Water-injected oats...	10	0	10	6
Control oats	10	0	10	0



Figure 3. *The experiment with oat plants, 9.6.-31.8.1959.* Left: plant injected with leafhopper extract. Right: control plant without injection.

of extract in all was injected into each LH-injected oat plant. Water was injected in the same way and in the same amounts. On 15.7., injections were stopped in the main shoots and begun in the tillers. Because of the small amounts of extract available, injections were only given every other day from 20.7. until the extract was exhausted on 12.8. A total of 1.7 ml. could be injected into the numerous tillers, and thus altogether 2.6 ml. per oat plant.

The effects of the LH-extract were already visible after 4 days, and after about 10 days the growth in length of the wilting main shoots ceased. With the LH-injected oats the height of the tillers also lagged behind that of the water-injected oats. On the other hand, the LH-injected oats had a great many more tillers than the water-injected oats. No tillers were formed on the control plants (Figure 3). The amounts of extract used did not suffice to inhibit panicle formation on all the tillers of the LH-injected plants. Table 2 shows the length growth and tiller formation, and Table 3 the number of panicles and seeds in each group.

The results show that the injection of LH-extract effectively inhibited the development of the main shoots but only to a lesser extent the growth in height of the tillers. It was also observed that daily injection of water inhibited the growth in height of the main shoot to some extent. This also appears from the decreased number of seeds in the panicles and from the increased number of tillers in comparison with the controls. Panicles were formed, however, on all the main shoots injected with water.

Table 2. *Growth in height and tiller formation in experiment 2.*

Material	Height of main shoots, cm.			Growth cm.	Number of tillers		Average height of tillers, cm.
	6.7.	19.7.	3.8.		3.8.	31.8.	
LH-injected oat a	15	18.5	18.5	3.5	4	4	22.5
" b	17	22	22	5	6	7	30
" c	13	17	17	4	5	8	18.5
Water-injected oat a	17	41	42	25	2	2	39.5
" b	10	28	34.5	24.5	1	1	56
Control oat a	15	47	49	34	0	0	
" b	13	44	47	34	1	1	31

Table 3. *The number of panicles and seeds on 31.8. in the oats in experiment 2.*

Material	Number of panicles		Tillers without panicles	Number of seeds	
	In main shoots	In tillers		In main shoots	In tillers
LH-injected oat a	0	2	2	0	25 + 15
" b	0	4	3	0	10 + 23 + 23 + 28
" c	0	5	3	0	8 + 12 + 10 + 8 + 8
Water-injected oat a	1	2	0	28	51 + 20
" b	1	1	0	18	54
Control oat a	1	0	0	48	
" b	1	1	0	65	26

Experiment 3 (7.5.-1.8.1959). In this experiment a single injection was given, as in experiment 1, but the plants were somewhat further developed. This was perhaps the reason why the damage was smaller than in experiment 1. In 17 LH-injected oats panicles were formed on the main shoots of 11 plants, the formation of panicles being inhibited in 6 plants. In a corresponding 17 water-injected oats, panicles were formed normally on the main shoots of all plants.

In none of the three experiments performed was the extract observed to cause any exceptional colouring of the plants. In addition, it was found that when spread on the stem the extract destroyed the epidermis but that it had no appreciable effect on the growth in height of the stem or on the formation of panicles.

Studies on the Chemical Nature of the Phytotoxin

When it had been demonstrated in these growth experiments that the factor in the water suspension of leafhopper extract was obviously the same as the factor causing damage to oat crops, a study of the chemical nature of this factor was begun.

In order to follow the growth-inhibiting factor, the straight-growth test on oat coleoptiles (Bonner 1957) was used. By means of this test the substance has been shown to remain in the lower layer and boundary surface when the water suspension is shaken with petrol-ether. From the water layer it is again clearly transferred to n-butanol when shaken with this. When the butanol is evaporated with addition of water, for the straight-growth test on coleoptiles, a profuse precipitate forms in the water. This can be separated almost completely with an ultracentrifuge.

When the fractionation of the butanol-soluble material from the leafhopper extract was started in the autumn of 1959, both the straight-growth tests on the coleoptiles and the growth experiments with the oats showed that the extract prepared from the leafhoppers collected in the area of oat damage in the growing season of 1959 unfortunately possessed only a small part of the effect of the corresponding extract prepared from leafhoppers in 1958. Likewise the oat fields in the western coastal area were found to have suffered less damage than in the previous year. In the growing season of 1960, hardly any damage could be observed. The extract from the leafhoppers injected did not have any effect and hence the leafhopper material then collected was unsuitable for further isolation experiments. The raw material used for the fractionation of the leafhopper extract was obtained by combining 10 ml. of the 1958 extract with 8 ml. of concentrated 1959 extract. The corresponding amounts of leafhoppers were 2.1 and 4.9 g. = 7 g. (about 7,000 specimens).

The fractionation of the leafhopper extract was carried out as follows. The combined extract was first shaken three times with petrol ether (a total of 50 ml. per 25 ml. of lower layer) and then five times with water-saturated n-butanol (a total of 55 ml.). The combined butanol solution (upper layers) was centrifuged. The amount of clarified solution was about 50 ml. None of the inhibitory effect of the leafhopper extract could be shown to have passed into the petrol ether. On the other hand, 50 or 60 per cent of the activity passed into the butanol, the rest of the effect remaining in the solid material which was insoluble in butanol. Water-saturated n-butanol served as solvent in the cellulose powder column, all fractions being of the same size. The amino acids emerged from the columns in fractions 12 to 14. The fractions were evaporated *in vacuo* at room temperature. The dry matter content of each fraction and the influence of them on the growth of oat plants are shown in Figure 4.

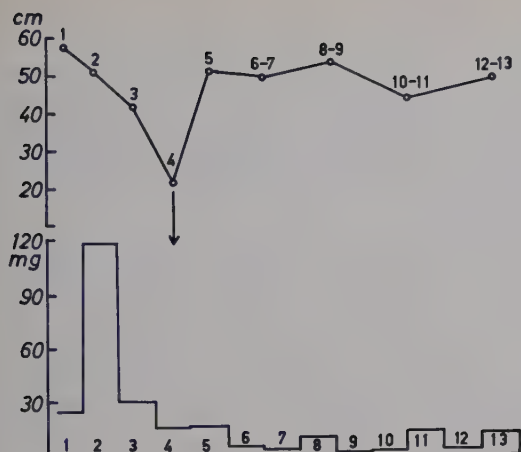


Figure 4. *Fractionation of a butanol extract of leafhoppers on a cellulose powder column and the effect of the fractions obtained on the growth of oat plants. Growth of oat plants in cm. and dry substance of different fractions of the butanol extract, mg.*

In the growth experiments with oats performed during the period 21.4.–15.7.1960, the fractions were injected into oat plants (3 specimens per fraction, except for fractions 6 to 13, where two fractions had to be combined because of their small size. The injection was performed in three portions, the total volume injected being 0.4 ml. The inhibitory effect appeared to be mainly concentrated in fraction 4 (Figure 4). Plants injected with this fraction reached an average height of only 21 cm. and no panicles were formed. Plants injected with the other fractions formed panicles without exception. Amino acids began to emerge from the column in fraction 12. As shown in Figure 4, fractions 12 and 13 had no inhibitory effect. Unfortunately, the straight-growth test with the coleoptiles gave inconclusive results because some fractions were infected, but the incomplete experiment indicated that the highest inhibition may have been exerted by fraction 4 too. This cannot be said for certain, however.

The dry weight of the fourth fraction was 15 mg. or 3.75 per cent of the dry matter in the total butanol solution. The substance was readily soluble only in ethanol and methanol. Attempts to crystallize it were unsuccessful.

Tests on Amino Acids and 3-Indoleacetic Acid in the Salivary Glands

Qualitative tests on the presence of amino acids and 3-indoleacetic acid were made with salivary glands dissected out from living leafhoppers in distilled water. After dissection the salivary glands were rinsed quickly in distilled water and placed in a solution of 70 per cent ethanol. Each sample studied consisted of 55 simple sali-

vary glands of females and 15 of males. The test animals of a sample from the area of oat damage were collected from oats on 3.7.1958 in the commune of Mustasaari. All the females in this sample were parasitized by Strepsiptera. A sample from outside the area of oat damage was collected from oats in the commune of Bromarf on 6.7.1958. One sample consisted of specimens which had been collected in Bromarf on 6.7.1958 and fed for 24 h on an artificial diet consisting of sucrose, l-histidine, l-glutamic acid and d-valine in aqueous solution. In paper chromatography tests 25 per cent of each sample was used.

Both the content of 3-indoleacetic acid and the occurrence of amino acids was investigated in all three samples. Samples of 5 ml. each were run through an Amberlite IR-120 column after which the column was washed with 70 per cent ethanol. The solution emerging was tested colorimetrically for 3-indoleacetic acid with $\text{FeCl}_3\text{-HClO}_4$ as reagent (Gordon and Weber 1951). Neither 3-indoleacetic acid nor related compounds were found in any sample by this method.

The IR-120 column was eluted with 1 *N* ammonia. The solution obtained was evaporated to dryness in a vacuum desiccator. One quarter of the residue was used for paper chromatographic detection of amino acids. On a two-dimensional paper chromatogram, with butanol-acetic acid-water and phenol-water- NH_3 as solvents, no clear spots except a weak one in the glycine position were formed on ninhydrin spraying. All three samples gave the same result in regard to both free amino acids and 3-indoleacetic acid.

Discussion

The disease symptoms in oat plants obtained in the present experiments by injecting the extract of *Calligypona pellucida* are essentially similar to the symptoms caused in oats by the oat sterile-dwarf virus (see *e.g.* the descriptions of Pruša *et al.* 1959; Vacke 1960; Lindsten 1960 a-b). The disease symptoms are also similar to those described by Kanervo *et al.* (1957) and Raatikainen and Tinnilä (1957) from the area of oat damage in Finland. The similarities in the symptomatology, as well as the positive correlation between the severity of oat damage in the field and the damaging effect of the leafhopper extracts obtained in the three different years, indicate that the disease symptoms obtained with the extract in the present experiments is caused by the same substance or substances as are responsible for the oat damage caused by *Calligypona pellucida* in the field.

The fact that the first step in the preparation of the extract consisted of the immersion and storage of the material in 70 per cent ethanol attests the chemical nature of the disease. The results presented in this paper afford

no answer to the question of how the active substances are formed in the leafhopper. The results thus do not exclude the possibility that the active substances extracted from the leafhoppers may have been formed by viruses in the insects. On the basis of the solubility of the active factors in butanol and their fractionation on a cellulose powder column, proteins and enzymes are excluded as active factors. Similarly, the negative results of the tests for the presence of amino acids and 3-indoleacetic acid in dissected salivary glands exclude these substances.

Summary

1. An ethanol extract of *Calligypona pellucida* injected into young oat plants greatly retarded their growth. No panicles were developed but numerous tillers were formed. Some of these developed panicles later on. No unusual changes in the colour of the leaves were observed. The results varied somewhat in different experiments, depending on the stage of growth at injection and, of course, on the amount of extract injected. Injection of water did not prevent the formation of panicles on the main shoots, although it slightly retarded growth in height, and promoted the formation of tillers to some extent. The difference between the plants injected with water and with extract was very great.

2. About 50—60 per cent of the active substance was dissolved in n-butanol from the ethanol extract. On fractionating the clear butanol solution thus obtained on a cellulose powder column, the active factors which inhibited growth and panicle formation was found to occur in fraction 4. Amino acids emerged from the column only from fraction 12 onwards. No inhibitory effect was found in fractions 12 and 13.

3. The results suggest that the growth-inhibiting factors in straight-growth test with oat coleoptiles was also responsible for the prevention of growth in height of the main shoot and panicle formation.

4. No amount of free amino acids worth mentioning was found in the salivary glands of *Calligypona pellucida*. Neither was 3-indoleacetic acid detected in these glands.

5. The disease symptoms in oat plants injured by the ethanol extract obtained from *Calligypona pellucida* were essentially similar to the disease symptoms caused in oats by the oat sterile-dwarf virus, which is known to be transmitted by the leafhopper in question. A positive correlation existed between the severity of damage to oats in the field and the damaging effect of leafhopper extracts obtained in three different years.

Our sincerest thanks are due to Mr. Teuvo Suominen and Mr. Raimo Hissa for carrying out the laborious task of collecting the leafhopper material from the area of oat damage during the year 1958 and to Mrs. Saara Kivinen, Miss Tuula Mäntyniemi, Mr. Matti Kivinen, and Mr. Pentti Kivinen for making the collections during the years 1959 and 1960.

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The Effects of Growth Substances on Attached and Detached Root Tips of *Pisum Sativum* L.

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Introduction

When isolated from the rest of the plant, tissues or organs are entirely dependent upon the medium; interacting mechanisms between different systems within the plant are eliminated and the environment is under complete control. This, however, is an artificial situation. Tissues and organs do not normally grow independently but are part of a complex physiological and morphological whole in which various organs and tissues interact, influence each other, and together comprise a morphogenic entity. What is of interest is how growth processes of the living whole plant differ from growth processes of parts of the living plant. Many studies have been confined to the use of intact plants; few have used excised plant systems.

Many investigators, using detached roots *in vitro*, have inferred that physiological and morphological effects of various treatments represent the reactions of roots *in situ*. But is this a valid assumption? It may be valid if the limitations of such detached systems are clearly defined and understood. Only then can information obtained from using detached organs be properly interpreted. These considerations may account for the apparent inconsistencies of observations on root length, cell elongation, and cell division.

The present study attempts to clarify these limits by determining if different effects on growth occur when completely detached roots and roots that are attached to the rest of the shoot are subjected to treatment with growth substances.

Materials and Methods

Seeds of *Pisum sativum* L. 'Alaska' were sterilized with 5.25 per cent sodium hypochlorite for five minutes and rinsed twice with sterile distilled water. The sterile seeds were germinated in darkness for 72 hours at 25°C. in Petri dishes containing one circle of filter paper moistened with 2 ml. of sterile distilled water. "Detached" root tips 5.0 mm. in length (Scheitler 1931) were excised and transferred aseptically to 125 ml. Erlenmeyer flasks containing 20 ml. of Torrey's medium (final pH 6.5) and grown in the dark for one week at 25°C. (Torrey 1954, 1956). Detached roots were harvested daily for one week and their final length measured to the nearest mm.

After sterilization, washing, and germination, "attached" pea roots from 20 to 23 mm. in length were marked at a point 5.0 mm. from the tip with a dot of India ink. They were placed aseptically on the top of 15 mm. × 120 mm. test tubes which were capped with aluminium foil. Torrey's medium was autoclaved separately and added to the tubes just before the immersion of the roots. A hole was made in the caps to allow the roots to be as completely immersed in the medium as possible. Samples were harvested daily for one week and the increase in length of the initial 5 mm. zone was measured.

Each growth substance, indole-3-acetic acid (IAA), kinetin (Kn) and gibberellic acid (GA), in Torrey's medium was used singly in the following concentrations: IAA, 1.2×10^{-4} M to 1.2×10^{-7} M; Kn, 5×10^{-4} M to 5×10^{-7} M; GA, 1×10^{-4} M to 1×10^{-7} M. Optimal concentrations of each of the growth substances as determined experimentally were then tested in all combinations.

After measurement, the root tip (from the India ink mark) was placed in 1 ml. of a solution of 5 % chromic acid and 1.2 N HCl (Brown and Rickless 1949), and macerated gently in a mortar which was washed with enough water to dilute the original suspension five fold. The suspension was vigorously shaken, 1 ml. was withdrawn into a tuberculin syringe and placed in a Sedgewick-Rafter Plankton cell counter. Counts were made with a 10 mm. objective and a 10 × ocular. A Whipple reticule in the ocular was adjusted so that the field covered by the reticule was one mm. squared. Ten successive fields were examined, moving from the left hand to the right hand edge of the counting chamber by strips at an angle to the long axis of the chamber. To calculate the total number of cells per ml., counts were multiplied by 5 (dilution factor), then divided by 10 (number of fields) and multiplied by 1000 (total number of fields in a chamber).

Each individual experiment consisted of 50 seeds, 10 as a control and 10 for each concentration of growth substances. Each experiment was repeated at least three times.

In determining significant differences between the means of controls and of treated roots, the following formula was used:

$$T = \frac{\bar{x} - \mu}{s/\sqrt{N}}$$

Significance was determined at $p = 0.05$ or $p = 0.01$. Some results were significant at the 5 % level and not at the 1 % level of confidence. For the sake of clarity, all references to deviations from the controls are at the 5 % level. The significance of the response of the root tips to each concentration of test substances was compared to that of the control of a given day.

Results

IAA

Attached pea roots

A. Length. A concentration of 1.2×10^{-4} M produced a complete inhibition of linear growth of pea roots throughout the experiment (Figure 1): 1.2×10^{-5} M suppressed elongation of the root and rate of growth; 1.2×10^{-6} M initially suppressed root elongation and growth rate, but by the sixth day an increase in both elongation and growth rate occurred. This accelerated rate of growth actually surpasses the rate of growth of the control. An initial suppression of root length and growth rate occurred at 1.2×10^{-7} M, but by the fifth day there was a similar reactivation as with 1.2×10^{-6} M. The accelerated rate of growth greatly exceeds that of the control.

B. Cell numbers. During the period of study there was a progressive increase in the number of cells in the untreated roots. Comparing the response of the roots in each concentration of IAA with its appropriate control on a particular day, there was no effect on the number of cells (Table 1).

C. Gross morphological observations. From day 4 to day 7, the controls formed extensive lateral roots and were morphologically normal. Gross abnormalities were observed at 1.2×10^{-4} M from day 4 to day 7. In many roots the steles split and lateral root primordia appeared. There was extensive browning of the roots, and characteristic "c-swellings" (McManus 1960)

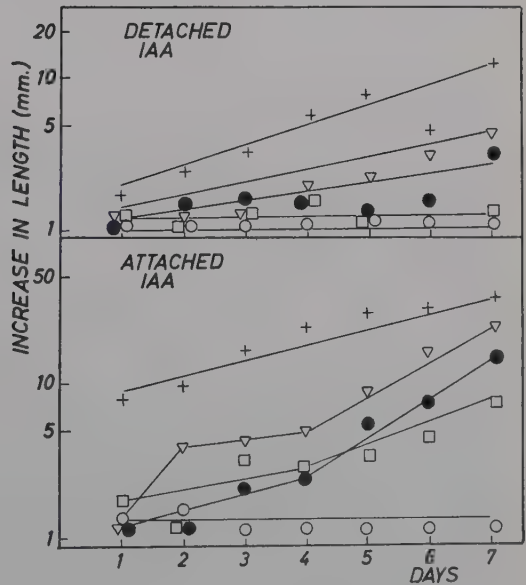


Figure 1. The effect of IAA on the elongation of attached and detached roots.

- \circ 1.2×10^{-4} M.
- \square 1.2×10^{-5} M.
- \bullet 1.2×10^{-6} M.
- ∇ 1.2×10^{-7} M.
- + Control.

Table 1. *The effect of IAA on cell numbers of attached (A) and detached (D) root tips.*
Total number of cells $\times 10^5$.

Concentration IAA <i>M</i>	Condi- tion	Days						
		1	2	3	4	5	6	7
0	A	25.5	38.6	35.5	39.1	45.1	40.5	28.0
	D	38.6	28.8	27.2	27.9	28.2	29.4	25.0
1.2×10^{-7}	A	36.7	35.6	36.0	33.2	39.0	30.9	29.0
	D	31.6	32.9	30.2	31.2	26.5	36.2 ²	35.3 ²
1.2×10^{-6}	A	35.6	35.6	31.3	31.1	27.0	31.5	33.1
	D	36.0	37.0	28.5	27.4	28.4	28.4	29.9
1.2×10^{-5}	A	31.0	27.1	30.6	29.6	41.7	29.7	28.9
	D	38.1	31.1	28.0	27.5	27.9	31.9	27.4
1.2×10^{-4}	A	30.4	31.3	28.0	29.9	36.4	41.1	25.2
	D	26.2	34.6	32.8	25.1	24.3	28.1	32.3

² pp = .01 %.

appeared. Abnormalities, characteristic c-swellings, and very small laterals emerging from the main root were observed at 1.2×10^{-5} *M* from day 4 to day 7. At 1.2×10^{-6} *M* some abnormalities were found, including c-swellings, and the amount of lateral formation was between that of 1.2×10^{-5} *M* and that of the control. From day 4 to day 7, c-swellings were found at 1.2×10^{-7} *M*; lateral root formation did not approximate the control until day 7.

Detached pea roots

A. Length. At 1.2×10^{-4} *M* root elongation and growth rate were inhibited. At the other concentrations, elongation and growth rate were much reduced, the lower concentrations causing less suppression than the higher (Figure 1).

B. Cell numbers. There were no differences in the numbers of cells found in control or those treated with any of the tested concentrations (Table 1).

C. Gross morphological observations. No abnormalities and no lateral root formation were observed.

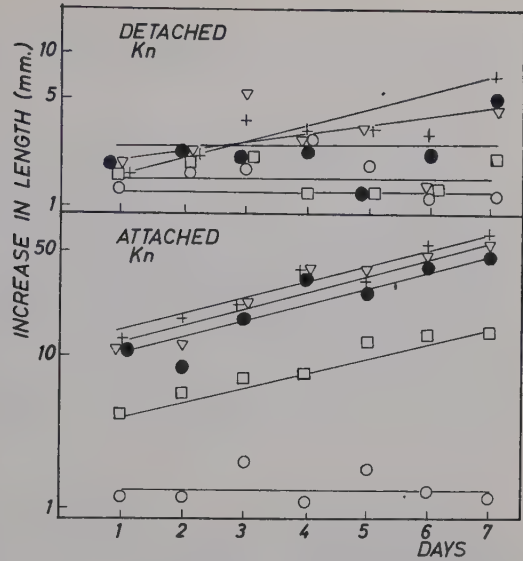
Kinetin

Attached pea roots

A. Length. At 5×10^{-5} *M* inhibition of root elongation (Figure 2) and growth rate was observed throughout the experiment. Suppression of root elongation and growth rate by 5×10^{-4} *M* Kn throughout the experiment was found. There was no effect on root elongation and growth rate at 5×10^{-6} *M* and 5×10^{-7} *M*.

Figure 2. The effect of Kn on the elongation of attached and detached roots.

- 5×10^{-4} M.
 □ 5×10^{-5} M.
 ● 5×10^{-6} M.
 ▽ 5×10^{-7} M.
 + Control.



B. Cell numbers. The number of cells in root tips treated with 5×10^{-4} M and 5×10^{-5} M Kn was less than that of the control. At 5×10^{-6} M and 5×10^{-7} M there were no differences in cell number (Table 2).

C. Gross morphological observations. At 5×10^{-4} M, from day 4 to day 7, there was inhibition of lateral root formation and some suppression of stem growth. The tips were very thick and showed extensive browning. 5×10^{-5} M also showed lateral root and stem inhibition with thickened tips and some

Table 2. The effect of Kn on cell numbers of attached (A) and detached (D) detached root tips. Total number of cells $\times 10^5$.

Concentration Kn M	Condi- tion	Days						
		1	2	3	4	5	6	7
0	A	45.7	48.7	44.9	51.6	51.7	57.0	44.9
	D	31.9	34.0	30.6	29.3	30.2	37.6	29.4
5×10^{-7}	A	47.7	45.3	50.8	49.2	49.0	57.1	49.1
	D	30.1	32.9	33.5	27.5	35.4	34.4	26.9
5×10^{-6}	A	44.8	38.1	42.5	46.4	46.3	55.2	47.7
	D	27.4	30.4	30.2	24.7	29.3	31.4	33.1
5×10^{-5}	A	37.3 ²	36.2 ²	34.1 ²	38.7 ²	35.1 ²	40.0 ²	38.8 ²
	D	27.5	27.9	31.2	30.0	29.1	33.4	28.7
5×10^{-4}	A	43.0 ²	29.7 ²	28.7 ²	27.2 ²	29.0 ²	29.7 ²	26.1 ²
	D	27.4	31.5	31.6	26.5	26.1	31.5	26.8

² pp = .01 %.

browning. At 5×10^{-5} M lateral root formation and stem growth by day 6 approximates those of the control. There was no difference from the control at 5×10^{-7} M.

Detached pea roots

A. Length. There was an inhibition of root elongation (Figure 2) and the rate of growth was lower than that of the control. The growth rate remained unchanged throughout the period of study.

B. Cell number. No change occurred in cell number with any concentration tested (Table 2).

C. Gross morphological observations. No morphological changes were observed.

Gibberellic Acid

Attached pea roots

A. Length. With 1×10^{-4} M GA, the elongation (Figure 3) and rate of growth of the tested roots were lower than the control. From day 1 to day 4 no difference between treated and control roots in root elongation and growth

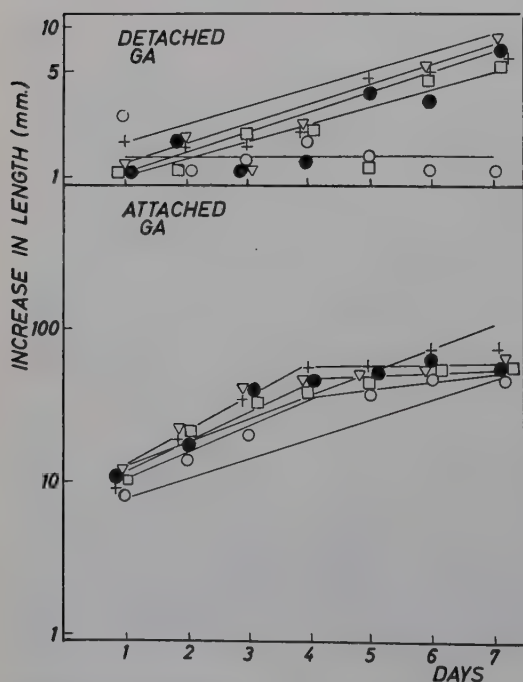


Figure 3. The effect of GA on the elongation of attached and detached roots.

- 1×10^{-4} M.
- 1×10^{-5} M.
- 1×10^{-6} M.
- ▽ 1×10^{-7} M.
- + Control.

Table 3. *The effect of GA on cell numbers of attached (A) and detached (D) root tips. Total number of cells $\times 10^5$.*

Concentration GA M	Condi- tion	Days						
		1	2	3	4	5	6	7
0	A	43.9	55.5	56.8	58.9	55.6	62.0	60.3
	D	41.2	37.9	41.6	36.9	33.2	35.2	39.2
1×10^{-7}	A	46.2	50.4	53.6	53.5	49.3 ¹	45.6 ²	53.6
	D	42.7	40.1	36.3	32.5	36.9	36.2	33.0
1×10^{-6}	A	39.0 ²	58.9	52.8 ²	48.8 ²	46.4 ²	49.1 ²	52.5 ²
	D	37.2	35.8	32.4	34.4	33.9	35.9	36.6
1×10^{-5}	A	36.6 ²	49.2 ¹	45.6 ²	44.1 ²	47.3 ²	48.2 ²	48.8 ²
	D	35.3	34.2	33.5	31.5	34.2	36.2	34.6
1×10^{-4}	A	36.5 ²	41.3 ²	39.8 ²	42.6 ²	42.6 ²	52.1 ²	58.2 ²
	D	39.3	38.6	32.4	33.7	36.4	32.3	34.1

¹ p = 0.5 %.

² pp = 0.1 %.

rate was observed at 1×10^{-5} M. At this time, a slight decrease in rate of growth and length of treated roots was noted. At 1×10^{-6} M again no difference from the control root elongation and growth rate was found until day 4 when there was a more pronounced and significant decrease in both.

B. Cell numbers. Fewer cells appeared throughout the period of experiment with the following concentrations: 1×10^{-4} M, 1×10^{-5} M, 1×10^{-6} M. No decrease was observed until day 5 at 1×10^{-7} M (Table 3).

C. Gross morphological observations. In general, lateral root and stem growth were the same as the control throughout the experiment. There were a few very thin and split root tips in the later days at high concentrations.

Detached pea roots

A. Length. With 1×10^{-4} M root elongation (Figure 3) and growth rate were inhibited. There was no difference from controls in root elongation or growth rate at 1×10^{-5} , 1×10^{-6} , and 1×10^{-7} M.

B. Cell number. There was no change in cell number at any of the tested concentrations.

C. Gross morphological observations. There were no morphological changes.

Mixtures of growth substances

Attached pea roots

A. Length. IAA at 1.2×10^{-7} M plus Kn at 1×10^{-7} M caused an inhibition of root length (Figure 4) and growth rate from day 1 to day 4. On day 4

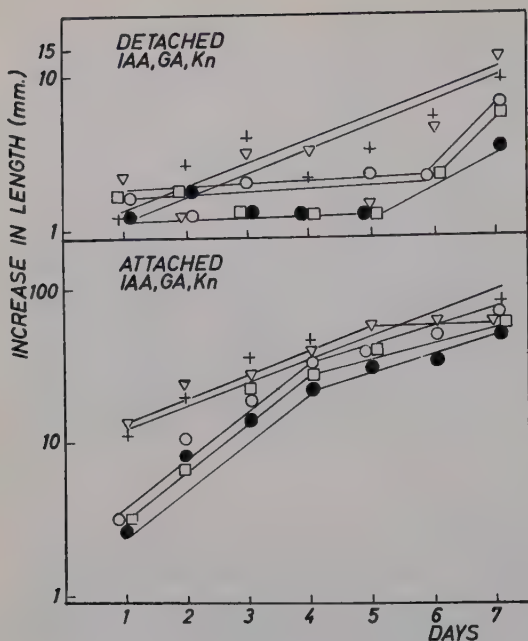


Figure 4. The effect of IAA, GA and Kn on elongation of attached and detached roots.

- IAA + Kn.
- IAA + GA.
- ▽ GA + Kn.
- GA + Kn + IAA.
- + Control.

there was an observable reactivation and increase of length and rate of growth; the rate of growth exceeded that of the control. The combination of IAA ($1.2 \times 10^{-7} M$) and GA ($5 \times 10^{-5} M$) produced the same pattern of growth as did IAA and Kn. The response to GA ($5 \times 10^{-5} M$) in the presence of Kn ($1 \times 10^{-7} M$) did not differ from that of the control until day 4, at which time there was a depression of root elongation with the growth rate falling below that of the control. The mixture of all three growth substances (IAA, $1.2 \times 10^{-7} M$; GA, $5 \times 10^{-5} M$; Kn, $1 \times 10^{-7} M$) inhibited root elongation and growth rate.

B. Cell number. There were no differences from the control in cell number in roots treated with IAA and Kn. Roots treated with IAA and GA show no change from control values until day 4 when the number of cells were decreased relative to those of the controls. The mixture of GA plus Kn and of all three showed fewer cell numbers than those of the control (Table 4).

C. Gross morphological observations. No morphological changes were noted.

Detached pea roots

A. Length. IAA in the presence of Kn caused a suppression of root length (Figure 4) and growth rate until day 6, when a sharp reactivation of rate

Table 4. *The effect of IAA, GA and Kn on cell numbers of attached (A) and detached (D) root tips. Total number of cells $\times 10^5$.*

Compound	Condition	Days						
		1	2	3	4	5	6	7
0	A	39.4	43.6	40.9	44.6	39.5	47.0	45.8
	D	38.7	34.7	33.7	32.7	34.9	33.3	36.5
IAA + Kn	A	38.4	40.8	42.9	45.7	38.6	40.2	46.6
	D	36.1	37.1	40.6	34.7	33.8	36.1 ²	46.6 ²
IAA + GA	A	34.1 ²	40.9 ¹	39.9 ¹	39.7 ²	39.7	39.3 ²	39.8 ²
	D	38.1	36.9	36.4	34.2	37.1	36.4	37.1
GA + Kn	A	38.5	37.5 ¹	38.8	39.0 ²	41.9	41.9 ²	41.3 ²
	D	34.7	37.5	33.9	35.1	35.0 ²	34.6 ²	36.3
IAA, GA + Kn	A	39.2	35.0 ²	40.9	40.4 ²	37.6	39.8 ²	41.7 ²
	D	37.4	33.9	35.9	33.3	33.6	33.3	36.3

¹ p = 0.5 %.² pp = 0.1 %.

and length occurred. These experiments were extended to 10 days, and the rate of growth, while greatly increased, did not equal that of the control. Initial inhibition was also noted with the mixture of IAA + GA; but during reactivation root length and rate of growth surpassed that of the control. The combination of GA + Kn did not cause any alteration from the control until day 6 when a very slight increase above control values was noted. The picture presented in roots receiving mixtures of all three was one of depression until day 6 when a slight reactivation occurred, but not enough to equal the length of the control.

B. Cell numbers. With IAA + Kn and IAA + GA, there was no change until day 5 and day 6 when an increase occurred. GA + Kn and mixtures of all three showed no change in cell number (Table 4).

C. Gross morphological observations. No abnormalities occurred, but there were two instances of lateral root formation in the mixture of IAA + GA.

Discussion

Indole-3-Acetic Acid

Levan (1939) concluded that auxin stimulated mitosis and enlargement in the meristematic region of the root tip of onion. Berger and Witkus (1948) found that a low concentration of NAA had no effect on this region of onion. In the zone of differentiation, where cell division had ceased, some cells were stimulated into cytokinesis. Their conclusions, in contrast to Levan's, were

that auxin stimulated division. Naylor and Rappaport (1950) found that, depending on the concentration, IAA could increase or suppress the length of pea roots in vitro or have no effect. Pilet (1951) found that auxin first stimulated and then suppressed the elongation of intact roots of *Lens*.

The ability to recover from the suppressive effects of IAA has been reported by numerous workers. Using detached pea roots, Audus and Das (1955) found that the maximum response to IAA was obtained in the first few hours; thereafter, the positive response declined. In intact wheat seedlings, Burström (1957) reported an initial inhibition of growth caused by added auxin, but in three days the inhibition was overcome and "adaptation" occurred. IAA had no effect on the rate of cell multiplication over a restricted period of time. He found that elongation proceeded at an increased rate as soon as the first inhibition was overcome. Adaptation to IAA was also observed in intact roots of *Allium cepa* by McManus (1960).

The nature of this reactivation is not yet clearly understood and several theories have been proposed to explain it. Galston (1956) believes that a cybernetic "feed-back" system exists between IAA and IAA-oxidase. When tissues are treated with IAA, their capacity to destroy IAA increases by the increased production of IAA-oxidase. This is similar to induced enzyme formation in microorganisms.

Pilet (1959) confirmed Galston's results with IAA and IAA-oxidase using detached roots of *Lens*. The kinetics of IAA destruction shows that degradation of IAA in young cells begins only after a lag period. If tissue extracts were treated with 2,4-dichlorophenol the lag period was absent, indicating that IAA-oxidase was already present in young cells, but that this enzyme system was blocked. Pilet postulated, in contrast to Galston, that the results are explicable in terms of enzyme adaptation or "induction" as defined by Cohn *et al.* (1953).

Burström (1957) does not think that an induced augmentation of IAA-oxidase activity is an adequate explanation for "reactivation". He postulated an alteration in the mechanism of cell extension involving other enzyme systems. These systems are probably concerned with the incorporation of cellulose in the growing cell wall. Burström (1953) classified four different actions of externally applied auxins; 1) a positive action on the first part of the cell elongation process, 2) an inhibitory action on the second part of the same process which composes the main part of elongation, 3) an anti-auxin action exerted by certain compounds, 4) an unspecified toxic action of both auxins and anti-auxins involving a reduction of the rates of both cell multiplication and cell elongation. The first two actions are genuine cell elongation effects not affecting cell multiplication; both are antagonized by anti-auxins.

Audus and Baksh (1960) concluded that "adaptation" or reactivation of roots to exogenous IAA occurred in the cells of the meristem before extension begins. This adaptation results from induced changes in the balance of enzyme complexes or of endogenous growth factors or both. These changes persist during subsequent extension and determine its pattern. Audus believes that with some substances a marked increase of IAA-oxidase activity may play an important role. However, a real change occurs in the sensitivity of what he calls "growth centers".

The results of the present investigation confirm previous studies in that the major action of IAA in the root is on the processes of cell extension. Both attached and detached roots were shorter but there was no positive effect on cell multiplication. Reactivation occurred only in attached roots indicating that during the first seven days of growth a complex interaction occurs between a substance located in other parts of the plant other than the root and exogenous auxin. Evidence has been presented suggesting that this substance is an uncharacterized enzyme (Tang and Bonner, 1947) or that it is IAA-oxidase (Galston, 1956; Pilet, 1959). It is doubtful that the substances are enzymes, since enzymes are rarely translocated. Perhaps reactivation is due to the inactivation of IAA as the indoleacetylaspatic acid complex. Detached root tips do not accumulate indoleacetylaspatic acid, whereas attached root tips do (Andreae and Van Ysselstein 1960 a, b). The complexing of IAA with aspartic acid is inversely proportioned to the concentration of exogenous IAA. Long-term experiments involving the treatment of roots with indoleacetylaspatic acid might shed some light on the reactivation of roots treated with IAA. The question of which system is involved in "reactivation" and how it operates has not been elucidated. It is fairly certain that this system does not exist or is inoperative in the detached root. Nevertheless, none of the available hypotheses can be entirely correct.

Kinetin

Kinetin has been described as a cell division factor, not as a cell elongation factor, although Miller (1956) found that cell elongation in pea stem segments was inhibited by Kn. Butcher and Street (1960) found that Kn decreased the production of new cells in the meristematic zone of excised tomato roots. Where growth of tomato roots was enhanced during the latter part of the growth period, there was a higher rate of production of new cells and longer mature exodermal cells. Guttman (1956) demonstrated that Kn in low concentration increased the number of onion root tip cells entering mitosis and also affected various stages of mitosis. McManus (1960) observed an inhibition of mitosis for a seven day period.

The available facts apparently lead to a paradox. The present studies on attached pea roots indicate that Kn inhibits cell division and does not promote it, although detached roots show no corresponding response. Several possibilities exist: a) a substance may have completely different actions in different parts or in different tissues of the same plant, b) Kn may affect both cell division and cell elongation, but cell elongation is masked by a greater capacity for cell division, c) the attached roots may contain a substance or substances that reinforces the inhibition of cell division by Kn, d) since the growth rate of detached roots is, in general, much slower than that of attached roots, an augmenting substance may not have accumulated, so that cell division is not affected by elongation.

Gibberellic Acid

It is fairly well established that the primary effect of GA on plants is to cause stem elongation, specifically by stimulating cell elongation. There is still some question as to whether cell division is also affected. Most of the reports indicate a definite inhibition of root growth (Stowe and Yamaki 1957). Brian *et al.* (1959) observed decreases in root weight of intact, treated peas and wheat. When GA was supplied to intact pea plants through the shoot, root weight increased. There are a few scattered reports to the contrary, (Richardson 1958). Pecket (1960) reported stimulation of root length of excised pea roots, but he made no cell measurements. There are some reports of increased cell division in shoots (Sachs and Lang 1957, Greulach and Haesloop 1958). McManus (1960) observed an initial depression of mitosis followed by a stimulation. In this study, too, the effect on cell division was one of depression and not of stimulation.

Interactions Among IAA, GA, and Kn

There is some evidence that interactions exist between IAA, GA, and Kn (Brian and Hemming 1957, 1958; Purves and Hillman, 1959). These reports indicated that most of the synergistic or additive effects of GA and IAA were obtained using detached parts of plants. Galston and Warburg (1959) have shown that in excised pea epicotyls growth is not additive when GA plus IAA is applied, but that growth effects are greater than additive if GA is applied basally to an excised segment and IAA is subsequently applied to an excised apical section. Even here the effect depended on where the GA or the IAA was applied to the detached segment. No synergistic effects have been found in attached plants. Purves and Hillman (1959) suggest that the primary action of GA and IAA is not closely connected. Skoog and Miller

(1957) found the mitotic rate of excised tobacco pith tissues was increased by appropriate mixtures of Kn + IAA. They concluded that the presence of IAA and Kn not only induced mitosis which set the stage for cell division, but also stimulated the latter process itself. Brian (1959) noted that responses to GA are obtained much more frequently from tissues with high endogenous growth rates. GA will induce a response in tissues with a low endogenous growth rate only if auxin is supplied. He thinks that the response to a high rate can be attributed to residual auxin. Fang *et al.* (1960) observed that GA inhibits the formation of indoleacetylaspate and results in an increased level of free IAA. This is in contrast to Pilet's results (1957) which indicated that the increase of free IAA + GA was a result of the inhibition of IAA-oxidase. However, the data of J. Kato and Katsumi (1958) on excised tomato roots support the conclusions of Brian and Hemming (1958). At low sucrose concentrations, where Kn inhibits growth, this suppression is intensified by adding GA. Suggesting the concentration of sucrose affects the balance between controlled growth factors of cultured root tip (Butcher and Street, 1960).

In the present study, there was no interaction of any kind on attached roots. Reactivation is due solely to the response of IAA. Tentatively, it could be postulated that some unknown substance in the attached root interacts with IAA and GA; it is absent from the detached root. The results on detached roots are not clear; apparently, some sort of interaction is occurring to cause reactivation. GA and Kn may compete with exogenous IAA for some other substance to prevent the complexing or inactivation of IAA in attached roots.

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Studies of the Growth in Culture of Excised Wheat Roots

II. The Growth-Promoting Activity of Amino Acids

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Introduction

Complex organic supplements rich in nitrogen, such as yeast extracts, peptones and hydrolysed caseins have previously been reported to enhance the growth of excised cereal roots (Kotte 1922, Robbins 1922, White 1932, Almestrand 1950) but in no case has the nature of the active constituents been determined. These supplements are all known to have, as major constituents, a range of amino acids. The experiments described in the present paper were, therefore, initiated to see how far the stimulatory effect of Bacto-casamino Acids upon the growth of excised roots of Atson wheat (Street, Carter, Scott and Sutton 1961) could be explained in terms of its amino acid content.

Materials and Methods

The source of root material and the general experimental techniques were as described in the previous paper of this series (Street, Carter, Scott and Sutton 1961).

Bacto-casamino Acids (Difco Ltd. B 230) is an acid-hydrolysed casein. The enzyme hydrolysed casein preparation referred to was Bacto-Casitone (B 259) of Difco Ltd. The mixtures of 13 (AA 13) and of 18 (AA 18) amino acids were to the composition and from the same samples of amino acids as described by Street, Hughes, and Lewis (1960) and were used at a concentration equivalent to 400 mg./l. Bacto-casamino Acids. The tryptophane samples were from Roche Products Ltd., the β -indolyl-acetic acid (IAA) from L. Light and Co. Ltd., and the gibberellic acid was a gift from Dr. P. W. Brian, F.R.S. of the Akers Laboratory of Imperial Chemical Industries, Ltd.

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Nitrogen determinations were carried out on a micro-scale by adaptation of the method 'Improved Kjeldahl method for nitrate-containing samples' of the Official Methods of the Association of Official Agricultural Chemists (8th Edition 1955, p. 12), combined with distillation of the digest in the Markham still and absorption of ammonia in boric acid. Extracts were evaporated to dryness at low temperature under vacuum in the Kjeldahl flasks prior to the digestion procedure.

Results

Comparison of amino acid mixtures with Bacto-casamino Acids

The mixture of 13 amino acids (AA 13) corresponding to the published amino acid analysis of Bacto-casamino Acids stimulated growth in both light and darkness but, in equivalent concentration, was less effective, under both conditions, than the casein preparation. Five further amino acids, quoted by Harrow (1946) in his amino acid analysis of casein, were added to this AA 13 mixture at concentrations based upon their content in casein to give the mixture of 18 amino acids (AA 18). The mixture AA 18 was superior in growth-promoting activity to the AA 13 mixture and *in light* fully replaced, at equivalent concentration, the activity of the Bacto-casamino Acids. These conclusions are illustrated by the specimen results in Table 1.

In a series of tests a number of simpler mixtures of the constituent amino acids of the AA 18 mixture were shown to be much less effective than the total mixture. Single amino acids, at their concentrations in the AA 18 mixture, either had no effect on growth or were significantly inhibitory as

Table 1. *The influence of Bacto-casamino Acids (400 mg./l.) and of equivalent concentrations of the amino acid mixtures upon the growth of excised wheat roots cultured in light and dark. Culture period 14 days.*

Treatments				Growth values			
				I.M.A.	L.N.	L.L.	Dry weight mg./15 roots
Dark Cultures	Basic medium	89	13	65	10.5
	"	"	+ Bacto-casamino Acids	116	31	342	23.2
	"	"	+ AA 13	84	16	97	13.9
	"	"	+ AA 18	85	17	176	17.2
	"	"	+ AA 18 + 26 mg./l. L-tryptophane	75	15	171	61.1
Light Cultures	Basic medium	183	14	137	40.7
	"	"	+ Bacto-casamino Acids	176	45	548	70.2
	"	"	+ AA 13	193	23	306	50.7
	"	"	+ AA 18	208	36	537	69.2
	"	"	+ AA 18 + 26 mg./l. L-tryptophane	48	9	103	63.8

additions to the basic medium (growth inhibition was noted with L-isoleucine, L-leucine, L-valine, L-histidine, L-lysine and L-threonine). A number of individual amino acids could be omitted from the AA 18 mixture with little or no consequent reduction of its growth-promoting activity but omissions of L-leucine, L-isoleucine, L-valine or L-arginine rendered the mixture growth inhibitory. Omissions of L-proline and L-cysteine significantly reduced the dry weight values of the cultures. These results strongly suggest the importance of a balanced mixture of amino acids. The inhibitory effect resulting from the omission of certain individual amino acids can be interpreted as due to the exposure of amino acid toxicities antagonised in the complete mixture by the omitted amino acids (Harris 1956).

One interpretation of the physiological activity of a balanced mixture of amino acids is that it acts as a preformed amino acid pool for protein synthesis. Its addition to the culture medium would then be expected to enhance growth in presence of nitrate if either the rate of nitrate assimilation or the synthesis of one of more essential amino acids were acting as limiting factors. This hypothesis receives support from nitrogen analysis of roots grown in nitrogen-omitted medium and media supplying nitrogen as nitrate, as Bacto-casamino Acids, as the AA 18 amino acid mixture, as Bacto-casamino Acids plus nitrate and as the AA 18 mixture plus nitrate. The nitrogen-omitted basic medium was prepared according to Sheat, Fletcher and Street (1959). Specimen results are presented in Table 2.

Table 2. *Growth and nitrogen content of excised wheat roots cultured in media containing various nitrogen sources.*

Treat- ment-	Nitrogen source			Growth values		Nitrogen values (mg. N)	
				I.M.A.	Dry wt. mg./10 roots	Total N 10 roots	Total N 100 mg. dry wt. roots
Dark	N-omitted medium			98	8.5	0.19	2.23
	" " + nitrate			86	9.1	0.39	4.28
	" " + Bacto-casamino Acids (400 mg./l.)			108	20	0.56	2.74
	" " + AA 18 amino acid mixture ...			110	14	0.36	2.65
	" " + nitrate + Bacto-casamino Acids			108	18	0.89	4.89
	" " + nitrate + AA 18 mixture			98	12	0.51	4.18
Light	N-omitted medium			128	13	0.18	1.39
	" " + nitrate			182	32	0.96	2.96
	" " + Bacto-casamino Acids			104	62	2.25	3.62
	" " + AA 18 mixture			101	36	1.03	2.88
	" " + nitrate + Bacto-casamino Acids			164	56	3.31	5.92
	" " + nitrate + AA 18 mixture			209	62	3.31	5.30

The nitrogen content of the initial radicle tips is not significantly lower than that of the roots developed during 14 days' incubation in the nitrogen-omitted medium; the nitrogen content of the 10 mm. tip alone permits of considerable root growth. Table 2 shows that roots cultured in presence of nitrate in the dark are rich in nitrogen but that no aspect of their growth is above that of the roots cultured in nitrogen-omitted medium. The dramatic stimulation of growth resulting from illumination is clearly associated in all nitrogen-containing media with increased nitrogen uptake per root and the beneficial effect of nitrate in the illuminated as compared with the 'dark' cultures suggests that light directly or indirectly may promote nitrate assimilation.

Bacto-casamino Acids is, as a sole nitrogen source, superior to the AA 18 amino acid mixture in both light and darkness, probably due, at least in part, to its content of non-amino nitrogen including ammonium or compounds readily yielding ammonium on hydrolysis (Street, Hughes, and Lewis 1960).

The beneficial effect of light although most marked in presence of nitrate is, nevertheless, highly significant as regards dry weight production by the cultures in nitrate-free medium (Table 2), and this effect is enhanced by some constituents of Bacto-casamino Acids not present in the AA 18 amino acid mixture.

The growth-promoting activity of 'autoclaved' tryptophane

The Bacto-casamino Acids contains per 400 mg., 54 mg sodium chloride and various B vitamins (in μg pyridoxine 0.03, biotin 0.05, thiamin 0.06, nicotinic acid 0.11, riboflavin 0.01). The growth-promoting activity of the AA 18 mixture of amino acids as a supplement to the basic nitrate-containing medium of 'dark' cultures was not enhanced by sodium chloride or a supplement of B vitamins, or both together to bring the level of these constituents to that resulting from the addition of 400 mg./l. Bacto-casamino Acids. The addition, in presence of the AA 18 mixture, of the volatile base distillable from the Bacto-casamino Acids at pH 9–10 or of this plus sodium chloride and extra B vitamins also failed to significantly enhance root growth. The superiority of the Bacto-casamino Acids over the AA 18 mixture in the dark was, therefore, not explicable in terms of the *known* non-amino constituents of the casein preparation.

The protein, casein, is rich in tryptophane but this may be expected to be destroyed by the acid hydrolysis involved in the preparation of Bacto-casamino Acids. The growth-promoting activity of 'autoclaved' tryptophane in contrast to the inactivity of the untreated amino acid, reported by Ro-

Table 3. *Effects of additions of Bacto-casamino Acids (acid-hydrolysed casein) and Bacto-Casitone (enzyme-hydrolysed casein) both at 400 mg./l. on the growth of excised wheat roots cultured in the dark.*

Treatment		Growth values			
		I.M.A.	L.N.	L.L.	Dry weight mg./15 roots
Basic medium		101	19	75	14.7
"	+ Bacto-casamino Acids (400 mg./l.)	121	30	347	28.2
"	+ Bacto-casitone (400 mg./l.)	107	24	251	37.2

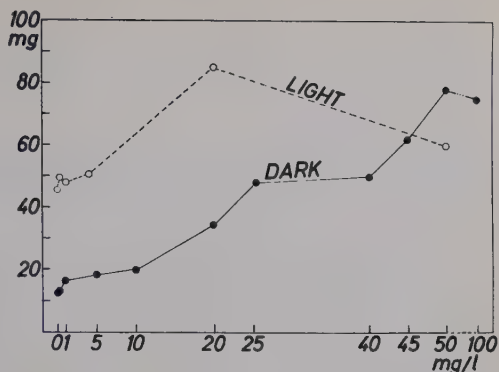
berts and Street (1955) in their studies with rye roots did, however, raise the possibility that even acid-hydrolysed casein might contain some derivative of this amino acid in physiologically active amount. In earlier work it had been observed that an enzyme-hydrolysed casein, known to contain tryptophane, when used at 400 mg./l. did, in dark cultures, enhance dry weight production even more effectively than Bacto-casamino Acids although it was less active in promoting linear growth of the cultures (Table 3). This led to studies involving the addition of L-tryptophane both in presence and absence of the AA 18 mixture of amino acids.

From the results in Tables 1 and 4 it can be seen that, in presence of the AA 18 mixture, additions of L-tryptophane to dark cultures very markedly enhance dry weight production but slightly inhibit linear growth. In the light, however, these tryptophane additions only enhance dry weight pro-

Table 4. *The influence of L-tryptophane additions to basic medium plus the AA 18 amino acid mixture (\approx 400 mg./l. Bacto-casamino Acids) on the growth of excised wheat roots cultured in light and dark.*

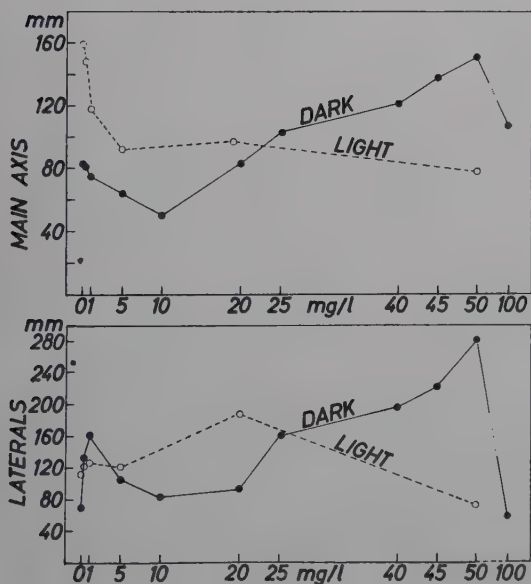
Treatment		Growth values			
		I.M.A.	L.N.	L.L.	Dry weight mg./15 roots
Dark	Basic medium	81	13	75	10.2
	" " + AA 18 mixture	100	21	174	19.0
	" " + " " " + 1.0 mg./l. L-tryptophane	84	20	225	18.2
	" " + " " " + 6.5 mg./l. "	70	17	199	25.3
	" " + " " " + 26.0 mg./l. "	69	20	207	45.5
Light	Basic medium	169	13	185	52.8
	" " + AA 18 mixture	200	38	554	76.4
	" " + " " " + 1.0 mg./l. L-tryptophane	196	35	538	84.5
	" " + " " " + 6.5 mg./l. "	80	9	121	64.5
	" " + " " " + 26.0 mg./l. "	44	8	63	63.7

Figure 1. The effect of additions of 'autoclaved' L-tryptophane (mg./l.) to basic medium upon the dry weight production mg. of 15 excised wheat roots during 14 days incubation at 26°C in light (900 lux) and in darkness.



duction at the lowest concentration tested (1 mg./l.) and, at the higher concentrations, strongly inhibit linear growth.

The growth effects of L-tryptophane additions to the basic medium are illustrated in Figures 1, 2 & 3. The dry matter accumulation of 'dark' cultures is progressively increased with increase in tryptophane concentration at least up to 50 mg./l.; in 'light' cultures the highest value for dry weight of roots was recorded with 20 mg./l. tryptophane (Figure 1). The addition of 50 mg./l. tryptophane results in the dark-grown roots having a dry weight equal to the highest value for the 'light' cultures (20 mg./l. trypto-



Figures 2 and 3. The effects of additions of 'autoclaved' L-tryptophane (mg./l.) upon the increase in main axis length (Figure 2) and the total length of laterals (Figure 3) of excised wheat roots cultured as indicated for Figure 1.

phane). All concentrations of tryptophane are inhibitory to main axis growth in 'light' cultures. By contrast, the higher tryptophane concentrations enhance the values for increase in main axis length of dark-grown roots bringing them close to the value for 'light' cultures in basic medium (Figure 2). Tryptophane enhances lateral initiation and growth in both light and darkness but the most marked enhancement occurs in the 'dark' cultures and the optimum tryptophane concentration is higher in the dark than in the light (Figure 3). These results indicate that tryptophane, at an appropriate concentration, is capable of enhancing all measured aspects of root growth *in the dark* to give values equal or above those achieved in basic medium in the light. It is also interesting to note that the degree of stimulation of growth caused by tryptophane and its most effective concentration are both lower in the 'light' than in the 'dark' cultures.

Attention should also be drawn to the relationship between tryptophane concentration and the linear growth values (Figures 2 and 3), particularly for the 'dark' cultures. The minimum growth values in presence of tryptophane occur at intermediate concentrations within the range tested. Riker and Gutsche (1948) in their studies of the amino-acid responses of sunflower crown gall tissue encountered a somewhat similar relationship between dosage and response. At very low and again at relatively high concentrations, the amino acids, glutamic and aspartic acids and alanine, stimulated growth but at intermediate concentrations (within the range 0.0001 to 0.002 M) they were strongly inhibitory. These workers postulated that these amino acids served a double function, suggesting that at low concentrations they had a catalytic effect on transamination and at higher concentrations acted as sources of nitrogen. Pollack (1943) in studies of the effects of α -methyl-pantothenic acid (a competitive inhibitor of pantothenic acid) on the growth of *Lactobacillus casei* observed, at first, increasing inhibition with increase in concentration but at still higher concentrations, growth was enhanced. Such results were interpreted as a dominance of the antimetabolite activity at low concentrations and ability to substitute in metabolism for the natural vitamin at sufficiently high concentrations. In considering our own data it may be important to bear in mind the evidence that 'autoclaved tryptophane' cannot be regarded as a single substance, and *in all the experiments described the L-tryptophane was autoclaved* with the other constituents of the medium. Our tryptophane sample was almost inactive when sterilised by filtration and subsequently added aseptically to cold sterile medium. Furthermore, autoclaved D-tryptophane when tested at 26 mg./l. was of similar activity to autoclaved L-tryptophane. These observations, in agreement with those earlier reported by Roberts and Street (1955) in their work with rye roots, indicate that the physiological activity of added tryptophane

Table 5. *The influence of β -indolylacetic acid (IAA) additions to basic medium on the growth of excised wheat roots cultured in the dark.*

Treatment	Growth values			
	I.M.A.	L.N.	L.L.	Dry weight mg./15 roots
Basic medium	101	8	47	14.0
" " + 50 mg./l. tryptophane	141	24	164	55.2
" " + 0.0001 mg./l. IAA	90	13	81	16.6
" " + 0.005 mg./l. IAA	68	12	90	21.2
" " + 0.025 mg./l. IAA	52	4	45	30.2
" " + 0.1 mg./l. IAA	41	3	27	45.5
" " + 0.2 mg./l. IAA	9	1	—	14.1

is dependent upon some change undergone by this indolic amino acid during autoclaving and that the natural isomer of the amino acid, which would be involved in protein synthesis, is not essential for activity. However, whereas Roberts and Street found β -indolylacetic acid (IAA) and the nitrile (IAN) to have activity comparable with activated tryptophane, these auxins do not reproduce the effects of tryptophane on the growth of wheat roots. Although these compounds at low concentration do enhance dry matter production by 'dark cultures' they are only inhibitory to linear growth as illustrated by the results with IAA presented in Table 5.

The method of Udenfriend and Peterson (1957) has been used to assess the indole content of wheat roots following extraction of the freshly harvested cultures with 80 % methanol. The indole content is expressed as tryptophane by reference to a calibration curve based upon pure amino acid. The values obtained, expressed as $\mu\text{g.}$ per g. fresh weight of roots were for roots cultured in basic medium in the dark, 0.01; in the light, 0.007; and for roots cultured in the dark in presence of 50 mg./l. tryptophane, 30.4. Light did not, therefore, enhance the total indole content of the cultures whereas addition of tryptophane to the culture medium established in the roots a relatively very high indole content.

The growth effects of gibberellic acid

Gibberellic acid can replace the light requirement for the germination of seeds of *Arabidopsis* (Kribben 1957), *Carnegieia gigantea* (Alcorn and Kurtz 1959), *Kalanchoë* (Bünsow and Seiferth 1959) and lettuce (Kahn, Goss, and Smith 1957). Both light and gibberellic acid promote leaf expansion and gibberellic acid can replace the long-day requirement for flowering of some plants (Lang 1959). In other cases, light and gibberellic acid seem to be opposed in their physiological effects. Gibberellic acid

antagonises the red-light inhibition of stem extension growth in *dark-grown* seedlings of pea (Lockhart 1956), *Perilla ocymoides* (Lona and Bocchi 1956) and *Helianthus* (Lockhart 1958). These results with etiolated plants have been interpreted by Lockhart and Gottschall (1959) as indicating a reduction of the endogenous gibberellin level on exposure to red-light. However, where light and gibberellic acid act in the same sense as in the promotion of stem elongation in light-grown long-day and certain other plants the results are frequently less than additive when both factors are used and this might suggest that the initial effect of enhanced day-length is the building up to the endogenous gibberellins to saturating levels.

Therefore, although it is at present difficult to bring the observed gibberellic acid-light interactions within a comprehensive hypothesis they do suggest that light either affects the level of or sensitivity to endogenous gibberellins. It was, therefore, decided to see whether our 'dark' and 'light' wheat root cultures would differ in their responses to external gibberellic acid. Further, the observation that tryptophane enhanced the growth of 'dark' cultures at concentrations inhibitory to 'light' cultures prompted us to examine if tryptophane and gibberellic acid treatments would show any marked interactions as reflected in the growth of our 'dark' cultures.

Gibberellic acid, over the range 0.01–1.0 mg./l. both as an addition to basic medium and in presence of Bacto-casamino Acids, does not signifi-

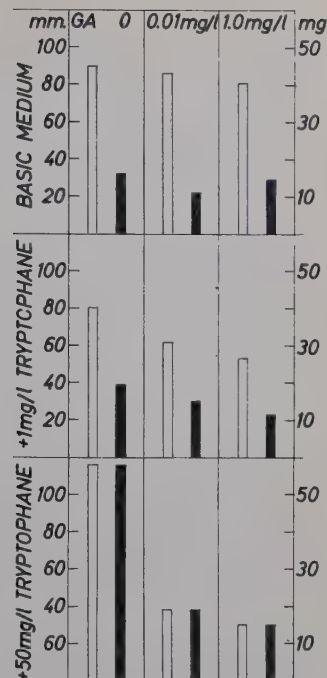
Table 6. *The growth effects of gibberellic acid (G.A.) upon excised wheat roots cultured in basic medium and basic medium + Bactocasamino Acids (400 mg./l) (GA) in light and darkness.*

Treatment				Growth values			
				I.M.A.	L.N.	L.L.	Dry weight mg./15 roots
Dark	Basic medium			84	12	57	12.9
	" " + 0.01 mg./l. G.A.			85	21	161	12.4
	" " + 0.10 mg./l. G.A.			86	26	182	12.5
	" " + 1.0 mg./l. G.A.			87	25	181	13.2
	Basic medium + C.A.			114	21	214	18.2
	" " + " + 0.01 mg./l. G.A.			111	27	338	18.8
	" " + " + 0.10 mg./l. G.A.			117	31	376	20.1
	" " + " + 1.0 mg./l. G.A.			120	34	443	20.8
Light	Basic medium			173	13	155	54.6
	" " + 0.01 mg./l. G.A.			87	3	19	15.5
	" " + 0.10 mg./l. G.A.			70	4	32	12.9
	" " + 1.0 mg./l. G.A.			65	6	32	11.9
	Basic medium + C.A.			155	30	384	78.7
	" " + " + 0.01 mg./l. G.A.			118	7	104	32.5
	" " + " + 0.10 mg./l. G.A.			97	6	123	22.6
	" " + " + 1.0 mg./l. G.A.			87	4	55	14.7

Figure 4. *The effects on main axis growth and dry weight production of excised wheat roots resulting from additions to the basic medium of 'autoclaved' L-tryptophane and gibberellic acid (G.A.) and mixtures of these two substances. Roots cultured for 14 days at 26°C in darkness.*

□ increase in main axis length mm.

■ dry weight mg./15 roots.



cantly affect main axis growth in 'dark' cultures but markedly enhances their lateral number and the extension growth of the laterals. The roots are characteristically finer and hence there is no associated enhancement of root dry weight. In strong contrast, gibberellic acid even at 0.01 mg./l. is markedly inhibitory to the growth of 'light' cultures, depressing their growth below that of the corresponding 'dark' cultures. Specimen results are presented in Table 6. Gibberellic acid at low concentrations can be seen to completely antagonise the beneficial effect on growth of illumination. It is, therefore, of interest that gibberellic acid also not only antagonises the beneficial effect of tryptophane (Figure 4) on the growth of 'dark' cultures but growth in cultures receiving both gibberellic acid and tryptophane (at concentrations at which each alone is stimulatory to growth) is below that of the control cultures in basic medium.

Discussion

The possibility of a light effect on nitrate assimilation raises again the question of its photochemical reduction (see Rabinowitch 1945) or the linking of nitrate reduction with photosynthesis (Burström 1943). Evans

and Nason (1953) have demonstrated a light dependent reduction of nitrate in presence of nitrate reductase, grana and catalytic amounts of triphosphopyridine nucleotide. In this connection, attention may be drawn to the apparently widespread formation of chlorophyll in illuminated roots, including those of wheat (Hejnowicz 1958). Subsequent work should, therefore, involve studies on both nitrate assimilation and chlorophyll formation in our cultures both in light and darkness.

The work with tryptophane suggests that further understanding of its activity and determination of whether light also acts through promoting the synthesis of some indole growth factor may follow a more detailed examination of the natural indoles of excised wheat roots. Wheat root cultures grown in light and darkness and in presence and absence of 'autoclaved' tryptophane will for this be submitted to extraction, solvent partition and chromatography by techniques previously applied to excised tomato roots (Thurman and Street 1960). Separation and identification of the active substance(s) in 'autoclaved' tryptophane will also be attempted by chromatography combined with a bio-assay procedure based upon excised wheat root cultures. Only when these lines of research have been explored can we hope to determine whether the observations with gibberellic acid really point to a similarity in the mechanisms of action of light and 'autoclaved' tryptophane in promoting the growth of our wheat root cultures.

Summary

Evidence is presented that the activity of Bacto-casamino Acids in promoting the growth of excised wheat roots is partly due to its balanced content of amino acids.

The presence of nitrate is of importance for the optimum stimulation of root growth by light.

'Autoclaved' tryptophane markedly enhances the growth of 'dark' cultures of excised wheat roots both as a single supplement to the basic medium and in presence of a mixture of amino acids. In light, tryptophane is inhibitory to main axis growth. Tryptophane enhances lateral initiation and growth in both 'light' and 'dark' cultures but the optimum stimulation is more marked and occurs at a higher tryptophane concentration in darkness than in light. Neither unheated tryptophane nor IAA, nor IAN have the growth effects of 'autoclaved' tryptophane.

Gibberellic acid, at concentrations which either do not affect or enhance the growth of 'dark' cultures in basic medium, is markedly inhibitory to 'light' cultures and to 'dark' cultures receiving stimulatory concentrations of 'autoclaved' tryptophane.

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Studies of the Growth in Culture of Excised Wheat Roots

III. The Quantitative and Qualitative Requirement for Light

By

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Introduction

Robbins and Maneval (1924) noted that light from a north-facing window, as against darkness, prolonged the duration of growth of excised maize roots subcultured every fourteen days. Later workers (Malyshev 1932, Fiedler 1936, Segelitz 1938, Delarge 1941), however, using excised roots of various maize varieties cultured under various nutritive and aeration conditions, reported light to be either almost inactive or inhibitory to growth. White (1932) seems to have observed some stimulation of excised wheat root growth by daylight and in artificial light reported a 20 per cent increase in the growth index of his cultures. Fujiwara and Ojima (1954) and Ojima and Fujiwara (1959) reported that light of 1000 lux from daylight fluorescent tubes was inhibitory to the main axis growth of excised wheat roots in a peptone-supplemented medium but without effect on growth in the basic-medium. Their results indicate that the inhibitory effect of the light decreased during the first 7 days of culture. Burström and Hejnowicz (1958) and Burström (1959, 1960) using high light intensities of 5,000–10,000 lux have reported growth inhibition and chlorophyll formation in excised wheat roots. Almestrand (1949) reported that light was without effect upon the growth of excised barley roots and inhibitory to the growth of oat roots.

Effects of light on the growth of the excised roots of a few dicotyledonous species have also been reported. Robbins (1940), working with excised

roots of *Datura stramonium*, noted that light was inhibitory to linear growth but that the roots growing in light were characteristically more robust. Gautheret (1935) found that light promoted the growth of excised roots of *Lupinus albus*. Roberts (1954) observed that diffuse light prolonged the duration of growth and enhanced apical dominance in roots of *Lycopersicum pimpinellifolium* repeatedly subcultured by excision of their main axis tips. Street (1953) found that low intensity light (4–97 lux) from tungsten filament lamps enhanced the rate and duration of main axis growth and retarded lateral development in excised tomato root cultures. Using gelatine colour filters, he obtained greatest activity in the orange-red although blue light was not entirely inactive. However, the filters used had broad transmission bands and the energy transmissions of the various filters were not equated.

It is clear that widely different light intensities have been operative in these various experiments with excised root cultures. In no case has a careful study of the effects of the intensity of illumination been carried out under otherwise rigorously uniform cultural conditions. Similarly, in no case has the action spectrum of the light effect been determined.

Reports that light has an inhibitory effect on seedling root growth go back to the work of Blaauw (1919) and it is clear from more recent papers that the effect can be observed with many species (Kohlbecher 1957). An action spectrum for this inhibitory effect of light on the growth of seedling roots of *Sinapis alba* shows some inhibition over the whole range 410–800 m μ with a peak of inhibition in the blue (ca. 450 m μ) and a much less marked peak in the red (660–750 m μ) (Kohlbecher 1957). Burström (1960) regards this spectrum as indicative of a porphyrin photoreceptor and stresses the possible relationship between the widespread formation of chlorophyll in illuminated roots (Hejnowicz 1958) and the growth inhibitory effect of light on root growth.

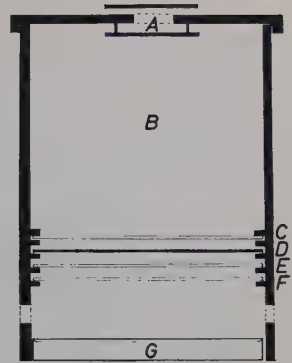
The marked enhancement, by light, of the growth of excised roots of Atson wheat, described in the first paper of this series (Street, Carter, Scott, and Sutton 1961) prompted the present study of the influence of light intensity and wave-length on the growth responses of these cultures.

Materials and Methods

The source of root material, the general experimental techniques and constituents of the culture medium were as described in previous papers (Street *et alia* 1961, Sutton, Scott and Street 1961).

All the experiments were carried out in a controlled-environment room maintained at 25°C. The temperatures of the experimental cultures did not exceed 26.5°C and

Figure 1. *Vertical section through a treatment box.* A = light-tight ventilator; B = culture compartment; C = clear glass plate $9'' \times 11 \frac{1}{2}''$; D = light-tight shutter; E = Ilford Spectrum filter; F = neutral density filter; G = perspex water screen resting on the diffusing screen.



variation in the temperature of the culture medium as between treatments in any one experiment was less than 0.5°C . The light source was a glass diffusing screen illuminated by a bank of 5' (80 watt) Osram internal reflector daylight fluorescent tubes at $5 \frac{1}{2}''$ centres. On this screen were placed the individual treatment boxes. These boxes (Figure 1) each housed up to twenty 100 ml. culture flasks standing on a clear glass plate $9'' \times 11 \frac{1}{2}''$, forming the base of the culture compartment 11" high and painted dull black internally. The light-tight lid of this compartment was perforated by a light-tight ventilator. Below the glass base of the culture compartment were housed the runners which could carry as required and in order downwards from the glass base, a light-tight shutter, an Ilford $10'' \times 12''$ spectrum filter (gelatin) mounted between glass, a neutral density filter and a clear perspex tray for a 1" water screen. Access to this light control compartment was by a hinged light-tight front. These boxes were placed upon a projection box (Figure 2) when the cultures were illuminated by the use of $2'' \times 2''$ Jena Spectralfarbfilters.

Neutral density filters for the treatment boxes were prepared from $10'' \times 12''$ Ilford N 30 plates by appropriate exposure to a cold cathode source (De Vere 54 Enlarger) followed by 4 min. development in Ilford ID 2. Neutral density filters for use with the Jena filters were similarly prepared from $3 \frac{1}{4} \times 3 \frac{1}{4}$ Ilford Contact

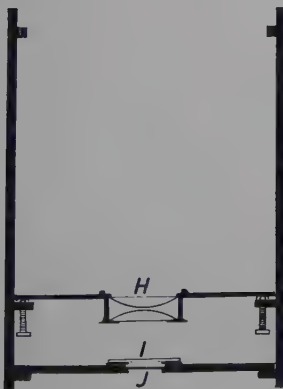


Figure 2. *Vertical section through projection box used with the $2'' \times 2''$ Interference filters.* H = condenser; I = neutral density filter; J = Jena Spectralfarbfiter $2'' \times 2''$.

Special Lantern Plates. Neutral density filters of the desired percentage transmission with the standard fluorescent source could be prepared by reference to a calibration of exposure time against optical density.

When illuminating with daylight fluorescent light directly or after transmission through the neutral density filters, intensities were determined at the glass plate of the treatment boxes by a Megatron type 'C' light meter and were expressed as lux. When working with the Ilford Spectrum filters or the Jena Interference filters their energy transmissions were measured in the dark room from a standard fluorescent light source using the Schwarz compensating linear thermopile FT 16 used in conjunction with a Tinsley mirror galvanometer type 4500 H (5 ohms resistance). Thermopile readings were used to calibrate a Mullard ORP 11 cadmium photoconductive cell used in conjunction with a micro-ammeter and a universal shunt. This photoconductive cell was then used to determine the operative experimental energy transmissions at the surface of the glass plate in the treatment boxes. Energy transmissions are expressed as erg./cm.^2 second.

The Unicam SP 500 Spectrophotometer was used to check the uniformity of transmission of the Neutral Density filters over the full spectral range and to determine the transmission characteristics of the interference filters.

Results

Light intensity

The maximum intensity used was 1500 lux and to study the influence of intensity this was reduced by Neutral Density filters used singly or in pairs. A water screen was not used in the experiments now described though other experiments, not now presented, but involving 1" water screens gave similar results.

The effects of light intensity on the mean values for main axis increase (mm.) and dry weight (mg.) per 20 roots are illustrated in Figure 3. Lateral number and total lateral length were enhanced along with main axis length and showed no independent trends. These values were reduced at maximum

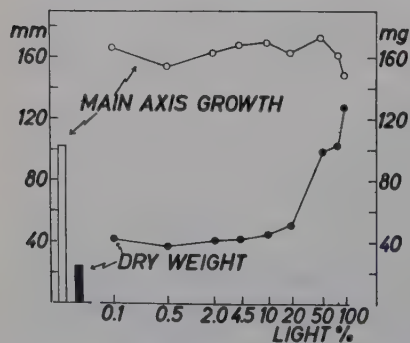
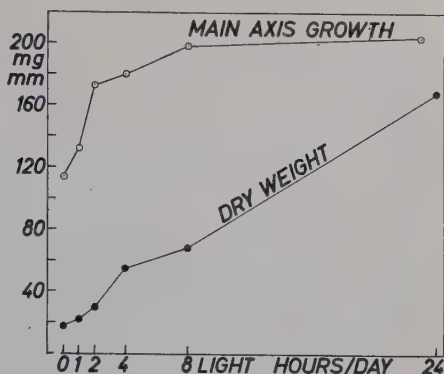


Figure 3. The influence of light intensity on main axis growth (mm.) and dry weight increase (mg./20 roots) of excised wheat roots cultured for 14 days at 26°C in basic medium. Light in per cent of full intensity (1500 lux). Histograms indicate growth of control 'dark' cultures.

Figure 4. Growth of excised wheat roots cultured for 14 days at 26°C in basic medium and illuminated for various periods, during each 24 hr. with light of 900 lux (60 per cent of full intensity). Main axis growth mm. and dry weight mg./20 roots.



intensity (100 %) as compared with 73 % intensity. From Figure 3 it can be seen that the full enhancement of linear growth was achieved at the lowest intensity tested (1.5 lux = 0.62 erg/cm² sec.). The values for dry weight did not rise significantly over the intensity range 1.5 to 150 lux but rose steeply with increase in intensity above 300 lux. These results suggest that two light effects operative at very different intensities may be involved.

This question of the possible existence of two light effects is also raised by experiments in which the roots were illuminated for periods ranging from 1–24 hr. each day at an intensity of 900 lux. The results of such an experiment are presented in Figure 4. The full enhancement of linear growth was only achieved when the light period reached 8 hr. per day. In a separate experiment roots receiving 20 minutes per day of light of 1500 lux showed no enhancement of linear growth above the 'dark' cultures (although this is equivalent in amount of light to 1.4 % full intensity continuously). Very much more light is therefore required for saturation of the effect on linear growth when it is interrupted by dark periods; intervening dark periods markedly reduce the effectiveness of light as a stimulant of linear growth. However, the steady rise in dry weight production of the cultures with increasing duration of light period suggests that the dry weight production was proportional to total light received and that its effectiveness was not reduced by the intervening dark periods.

Light quality

Action spectra have been obtained using the Ilford spectrum filters as indicated in Figure 5 and also using the Jena Interference filters (Figure 6), transmission data for which, based on our determinations, are summarised

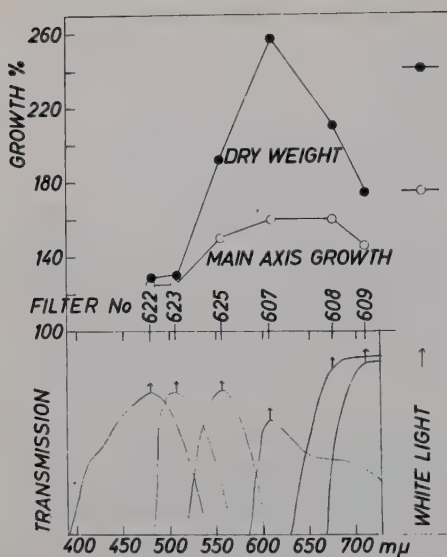


Figure 5. Transmission curves for selected Ilford Spectrum and Bright Spectrum filters and growth of excised wheat roots (14 days at 26°C) receiving light transmitted by these filters and adjusted for each filter to 10 ergs/cm²·sec. by appropriate neutral density filters. Light source: Osram daylight tubes (80 watt) filtered through 1" layer of water. Growth values as per cent of dark controls plotted against peak transmission wave-length of each filter.

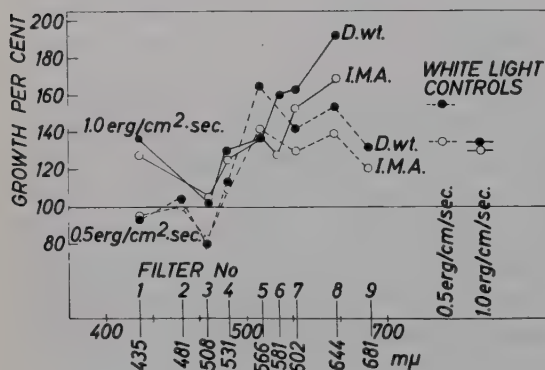


Figure 6. Growth of excised wheat roots (14 days, 26°C, per cent of dark control) in radiation transmitted through Jena Spectralfarb-filters (transmission characteristics shown in Table 1) at two energy levels, viz. 0.5 and 1.0 ergs/cm²·sec. Growth values plotted against peak transmission wave-length of each filter.

Table 1. Transmission characteristics of Jenaer Glaswerk Spektralfarbfilters as determined on the Unicam SP 500 Spectrophotometer.

Filter No.	λ max. mμ.	Band width at 10 % transmission mμ.
1	435	426 — 446
2	481	475 — 489
3	508	495 — 518
4	531	520 — 543
5	566	554 — 582
6	581	570 — 595
7	602	591 — 618
8	644	632 — 659
9	681	671 — 692

in Table 1. Water filters (1" layer) were used only with the Ilford filters. Appropriate neutral density filters were prepared to obtain uniform total energy transmissions in ergs per $\text{cm}^2 \cdot \text{second}$ through the filters and in the 'white light' controls. This means that more quanta were received in the red than in the blue but, nevertheless, from the contrast in activity between these regions, it can be predicted that similar action spectra would have been obtained had transmissions been standardised on a quantum basis. It should further be emphasised that if light has two distinct effects upon root growth then the action spectra now reported apply only to that effect which is dominant at low intensities. It is an essential part of future work to use a more intense light source in conjunction with the Interference filters.

The results clearly indicate a broad peak of activity in the region 580–650 m μ . This contrasts with the greatest activity in the blue region reported by Kohlbecher (1957) for the inhibition of seedling root growth in *Sinapis alba* and suggests the possible functioning of a photoreceptor of the type involved in the red light effects on seed-germination, internode inhibition and photoperiodism. It is, therefore, necessary to examine critically whether this red light promotion of wheat root growth in culture is reversible by the appropriate application of far-red light.

Discussion

The very low light intensity which, applied continuously, achieves maximum stimulation of linear growth of our cultures and the marked extension of the duration of growth in illuminated cultures (Street *et alia* 1961) raise the question of how far excised root cultures in general have an essential light requirement. No-one has, to our knowledge, carried out a prolonged subculture experiment under conditions approaching complete darkness. This should certainly now be attempted with tomato and other species whose excised roots maintain a high rate of growth under the usual conditions of culture which involve intermittent exposures to light.

Studies on the persistence of meristematic activity in repeatedly subcultured root tips of tomato (Street, McGonagle, and Roberts 1953, Street 1954) have emphasised that treatments (increase in sugar concentrations, application of auxins) which enhance the percentage dry matter of the roots lead to 'ageing' and ultimate loss of function in the meristems. Therefore, before concluding that wheat roots have a further growth requirement not met in illuminated cultures growing in our basic medium supplemented by Bacto-casamino Acids, it will be necessary to study further their be-

haviour on repeated subculture using light intensities far below that (900 lux) used in the experiment reported by Street *et alia* (1961).

The effects of relatively low light intensities over comparatively long periods (14 day passages) on excised wheat root growth described in this and preceding papers are not, at this stage, easy to compare with the interesting studies of Burström and his colleagues on the growth inhibition observed at higher intensity and over shorter periods. Attention has already been drawn to the existence of a phase of inhibition followed by a phase of growth enhancement during the first 14 days growth in culture of the radicle tip (Street *et alia* 1961). In the present paper, attention is drawn to the evidence that our intensity of 1500 lux is supra-optimal for overall linear growth during 14 days culture. It is, therefore, important now to extend our studies by examining cell expansion and cell division during the time course of growth in 'dark' and 'light' cultures and to see how far illumination at our low intensities is accompanied by chlorophyll formation in the roots.

Summary

Continuous fluorescent illumination at an intensity as low as 1.5 lux (0.62 ergs/cm² sec.) can cause full expression of the enhancement of the linear growth of excised wheat roots by light. A marked increase in the percentage dry matter and robustness of the cultures occurs at an intensity of 300 lux or higher. The effectiveness of light in enhancing linear growth is reduced by intervening dark periods.

Action spectra of the light effect determined at intensities ranging from 0.5 to 10 ergs/cm². sec. show a broad peak of activity from 580–650 mμ.

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Biological Phosphorylation of 3-Amino-1,2,4-Triazolyl-glucoside in *Cryptococcus neoformans*

By

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Introduction

Despite the fast-expanding literature on 3-amino-1,2,4-triazole (3AT), the physiological action of this phytotoxic heterocycle still remains, for the most part, undefined. Its interference with the metabolism of amino acids, specifically glycine and serine, has been reported (Carter and Naylor 1961). Hilton (1960) found that the inhibition of the multiplication of several yeasts caused by 3AT could be reversed by the addition of L-serine to the growth media. Massini (1959) isolated what he believed to be a detoxified form of 3AT, 3-amino-1,2,4-triazolylalanine. This amino acid conjugate of 3AT has recently been suggested (Carter and Naylor 1961) as arising from the reaction of phosphoserine and the 3-amino group of 3AT.

Interference with carbohydrate metabolism has been reported by Rogers (1957), and by McWhorter and Porter (1960). The last two investigators found that carbohydrates were catabolized very slowly by 3AT-treated plants. Possible interference with glycolysis has been reported by Fredrick and Gentile (1960 a) as arising from the inhibition of phosphorylase by 3AT. This inhibition, at first thought to be due to the chelate-forming abilities of 3AT (Sund 1956), was subsequently ascribed to the formation of a glucose derivative of 3AT by the reaction of 3AT and the phosphorylase substrate, glucose-1-phosphate (Fredrick and Gentile 1960 b). The amine glucoside (GAT) formed had been observed as being common to plants treated, in vivo, with 3AT

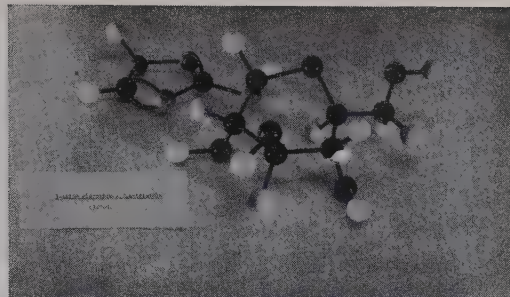


Figure 1. Molecular model of the glucoside of 3-amino-1,2,4-triazole. Triazole nucleus is to the left of the photograph; the glucose residue is on the right. Note the amine linkage.

(Sund 1956). The structure of GAT has recently been established (Fredrick and Gentile 1961, 1960 c). A molecular model of GAT is shown in Figure 1.

GAT can be utilized by hexokinase (Gentile and Fredrick 1959). Although it has been reported to have only approximately fifty per cent the affinity for this enzyme that glucose has, its use by hexokinase raises interesting questions as to its possible metabolic fate. For example, if GAT is phosphorylated by this enzyme, does it undergo further hydrolysis via an enzymatic route such as by some type of β -glucosidase, and is free 3AT liberated thereby?

GAT has been shown to be extremely stable, even under strong acid conditions (Gentile and Fredrick 1959). Therefore, its possible phosphorylation by hexokinase could conceivably render it susceptible to enzyme action by other plant enzymes.

The present studies were undertaken with a view to answering these questions. Use has been made of a recent yeast isolate, a non-pathogenic form of *Cryptococcus neoformans* (Benham 1955, Fredrick 1961 a). This organism grows well on simple agar containing glucose as C source and potassium nitrate as N source (Fredrick 1961 a). The yeast forms starch (Benham 1955), therefore indicating a phosphorylative glycolytic mechanism present.

Experimental

Cryptococcus neoformans var. *innocuous* Benham was maintained on 2 % agar (Difco "Bacto-agar") containing 0.1 M glucose and 1 % potassium nitrate. The pink mucoid growth was luxuriant after 48 hours at 24°C (Fredrick 1961 a). The yeast was removed from the agar surface by means of an inoculating loop and suspended in isotonic saline. It was washed twice with saline, centrifuged, and then resuspended.

Three series of streak plates were made up with the washed yeast. The control series consisted of 1.5 % agar containing 0.1 M D-glucose and 1 % potassium nitrate. A second series contained the same ingredients except that 0.1 M GAT was used in place of the D-glucose. The GAT was prepared and purified as described previously

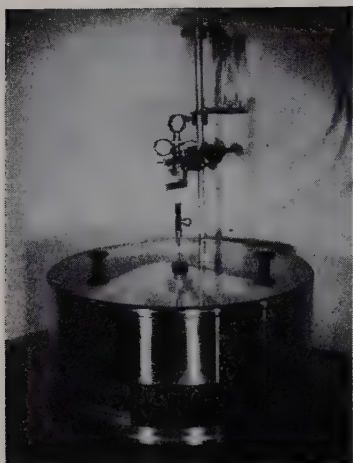


Figure 2. *The centrifugally-accelerated chromatography apparatus.* Complete descriptions of its operation and the techniques involved, have been published previously (Fredrick 1961, 1959).

(Gentile and Fredrick 1959, Fredrick and Gentile 1960-c). The third series of petri dishes contained the control media plus 0.1 *M* recrystallized 3AT (Gentile and Fredrick 1959).

After 48 hours incubation at 24°C, the yeast was collected from the GAT series of dishes, washed in saline, centrifuged, drained, and, macerated in a Kontes Disintegrinder fitted with a Teflon pestle. The resulting homogenate was extracted with five times its volume of boiling water, filtered through cheese-cloth and then, cooled in the refrigerator for 3–4 hours. Cold 5 % trichloroacetic acid was added to precipitate the proteins and the extract was filtered through Whatman no. 54 paper. The filtrate was adjusted to a neutral pH with dilute ammonium hydroxide, and then concentrated by boiling down to one fourth its original volume.

The concentrated filtrate was chromatographed by high speed centrifugally-accelerated paper chromatography using a Hi-Speed Chromatograph (Precision Scientific Co.) as described by Fredrick (1961 b). This apparatus is shown in Figure 2. The developing solvent was that used by Fredrick (1961 b), the water-saturated butanol layer of a 4 : 1 : 5 mixture of *n*-butanol, acetic acid and water. The chromatograms were developed at a speed of 1720 r.p.m. for 6 minutes (see Fredrick 1961 b).

The chromatograms were air-dried and then, sprayed with a solution of Sund's modified nitroprusside reagent (Sund 1956). With this spraying reagent, 3AT appears as a bright green spot, GAT as a violet-pink spot, and the phosphorylated GAT as an orange-red spot. The R_f values of these compounds have been reported to be 0.14 for GAT, 0.50 for 3AT, and 0.31 for the unknown or phosphorylated GAT (Fredrick 1961 b).

The presence of labile phosphorus in the R_f 0.30 spot was confirmed by spraying an extract-derived chromatogram with acid-molybdate and fuming with hydrogen sulfide (Fredrick 1961 b). Ester phosphorus is detectable by this reagent (Hanes and Isherwood 1949).

Results

Figure 3 shows a streak plate from each of the three series described. Note that 3AT has completely inhibited the growth of the yeast in spite of the fact that an optimum concentration of D-glucose was present. It is of interest, that GAT did not form in this series under these conditions despite the mole/mole relationship of D-glucose and 3AT.

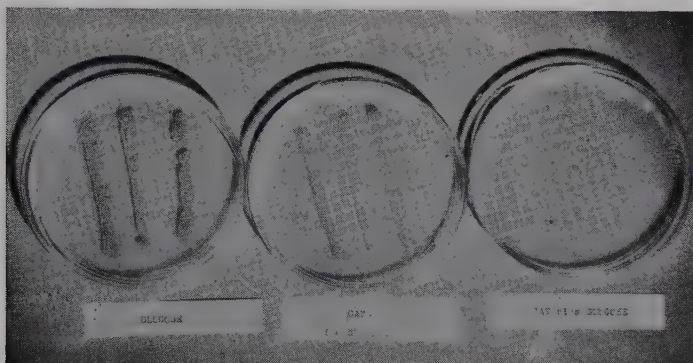


Figure 3. Growth of *Cryptococcus* on D-glucose, the amine glucoside and on D-glucose with 0.1 M 3-amino-1,2,4-triazole added. Note the complete inhibition of growth by 3AT (right) in spite of an optimum glucose concentration. The growth of the yeast on the amine glucoside in the absence of glucose (middle) is sparse as compared to its growth on D-glucose (left).

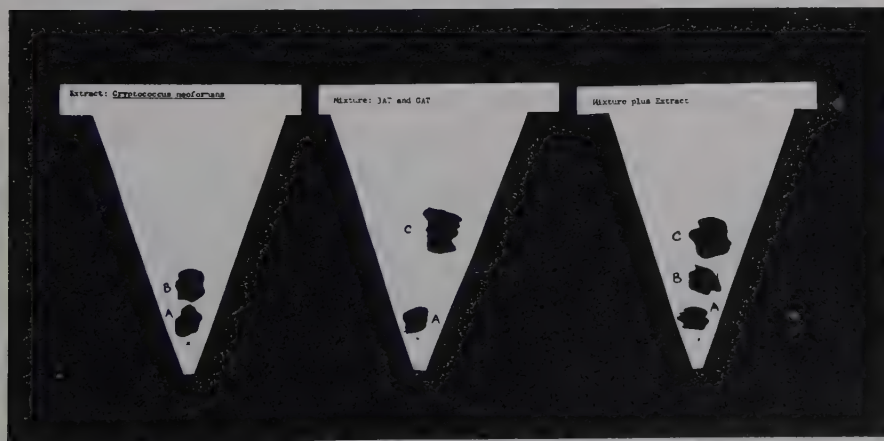


Figure 4. Radial chromatogram sections of the extract from the yeast grown on the amine glucoside (left) as compared to artificial mixtures of the glucoside and 3AT, and of the artificial mixture plus added extract (right). Sprayed with Sund's nitroprusside (Sund 1956).

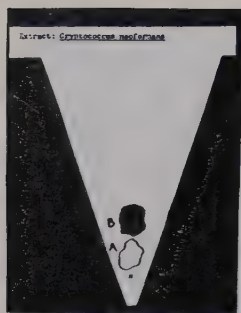


Figure 5. Chromatogram derived from extract (as in left, Figure 4) but sprayed with a reagent to make visible phosphoric esters. Spot B is in the same position (R_f 0.30) as that in the chromatogram in Figure 4. Spot A has been outlined using the chromatogram in Figure 4 as a guide to show the approximate position of GAT. The acid-molybdate reagent only causes the spot at B to appear.

The middle plate of this figure (Figure 3) shows the growth of the yeast on GAT; this growth is apparently less than when D-glucose is used as the carbon source (left, Figure 3).

Figure 4 shows sections from a radial chromatogram. Note that the *Cryptococcus* extract from the yeast grown on GAT shows only two spots, that for GAT (at R_f 0.14) and that for phosphorylated GAT (R_f 0.30). No spot is present which would indicate free 3AT in this extract. That such combinations may be detected can be seen from the two other chromatograms in this figure. Mixtures of 3AT and GAT as well as with the extract itself, all give clearly discernible separations.

Figure 5 shows a duplicate chromatogram of Figure 4, left. In this case, however, the acid-molybdate has been substituted for the nitroprusside as the spray reagent. Note that only one spot appears, at R_f 0.30. For comparison purposes, the relative position of the GAT spot has been outlined, using the data from Figure 4.

Discussion

Cryptococcus had a limited growth on GAT (cf. Figure 3, left and middle plates) as compared with its growth on d-glucose. When extracts of this yeast were chromatographed, only two spots appeared (Figure 4, left, and Figure 5). These spots were at R_f s of 0.14 and 0.30. When the chromatograms were treated so that ester phosphorus-containing compounds were made visible, only one spot (R_f 0.30, Figure 5) appeared. It would seem that GAT is phosphorylated by this yeast, the phosphorylation probably being due to the enzyme, hexokinase (Gentile and Fredrick 1959).

At no time was free 3AT detected in the chromatograms of these extracts from GAT-grown *Cryptococcus*. Hence, GAT is probably directly phosphorylated, and therefore does not undergo hydrolysis prior to hexokinase action. It

is probable that phosphorylation occurs at the number 6 carbon of the glucose residue of this compound. This, based upon our knowledge of the more or less non-specific requirements of hexokinase for glucosidic substrates, is entirely possible.

Of interest, is the fact that unchanged GAT still appeared in these extracts (Figures 4 and 5), together with phosphorylated GAT. This indicates that the phosphorylation of GAT is probably a slow enzymatic process, and therefore, substantiates the previous observation that GAT has less of an affinity for hexokinase than does D-glucose (Gentile and Fredrick 1959).

McWhorter and Porter (1960) have reported an increased fat content in seeds attached to 3AT-treated plants. They attributed this to a conversion of carbohydrate to fat in these plants. Sund (1956) had reported that GAT was common to all plants treated with 3AT. In view of these reports, it seems possible that GAT is phosphorylated by hexokinase so that it is rendered more labile to subsequent enzymes' actions. The amine glucoside is, in itself, an extremely stable structure (Gentile and Fredrick 1959, Fredrick and Gentile 1961).

The ultimate fate of the phosphorylated GAT is of interest. If the compound is broken down by plant β -glucosidases, free 3AT should be liberated, and therefore, detected. No such traces of the free aminotriazole were detected in these studies (Figure 4), and, therefore, at least in this variety of the yeast, no such conversion occurs. This problem is currently being explored.

Conclusions

The pink yeast, *Cryptococcus neoformans* utilizes the glucoside of 3-amino-1,2,4-triazole in place of d-glucose. Growth of this yeast on the glucoside is less than on glucose. The chromatographic analysis of extracts prepared from *Cryptococcus* grown on the glucoside shows evidence of the phosphorylation of the amine glucoside. At no time was free 3-amino-1,2,4-triazole detected in these extracts. The possibility of the further metabolism of the phosphorylated amine glucoside is discussed with a view as to delineating the ultimate fate of the phytotoxic heterocycle, 3-amino-1,2,4-triazole.

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The author is deeply indebted to Mr. Albert Sanchez for the illustrations used in this study.

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Longitudinal and Lateral Response of Etiolated Pea Sections to Indoleacetic Acid, Gibberellin, Kinetin, Sucrose, and Cobaltous Chloride

By

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Elongation of etiolated pea sections in response to treatments with indoleacetic acid (IAA) and gibberellin (GA) has been studied frequently (1, 3, 4, 5, 12). Where reactive tissue is located and how sucrose and cobaltous chloride affect elongation have been reported (4, 8, 12, 16). Recently, certain paper chromatographic fractions of an ethanolic extract from cultures of the fungus *Taphrina deformans* (Berk.) Tul. were shown to inhibit elongation in response to IAA while causing a striking expansion in diameter. That expansion appeared due, in part, to stimulation of cell division. In this respect, the action appeared similar to that of kinetin (KN), and the presence of a kinin is suspected (15).

Bioassays with tissue-culture techniques for substances that stimulate cell division are well known (9, 10). More rapid bioassays with various leaf tissues use cell enlargement (6) or delayed senescence (11) as an index of KN or kinin activity. Cell division is evidently not stimulated in these leaf tissues (6, 11, 13). The reaction of pea sections to *Taphrina* extracts and KN suggests that they may provide a rapid bioassay, involving cell division, for use with paper chromatograms.

An investigation was made to determine the effect of KN, GA, IAA, sucrose, cobaltous chloride, and combinations of these, on elongation (longitudinal response) and, more particularly, increased diameter (lateral expansion) of etiolated pea sections. A related consideration was evaluation of the possible usefulness of such sections in bioassays of substances that stimulate cell division.

Materials and Methods

Pisum sativum L. (variety Alaska) seeds were soaked overnight in running tap water and planted in wet vermiculite in polyethylene pans of 8 by 11 by 6 inches. Humid atmospheres were ensured without large changes in atmospheric oxygen or carbon dioxide by enclosing the pans within polyethylene-film bags having 12 ventilation holes of $\frac{1}{4}$ -inch diameter. The seeds were grown for six days at $20 \pm 1^\circ\text{C}$ in total darkness except for brief exposure to weak red light during manipulations.

Sections of seedlings, cut with a tool consisting of parallel razor blades held 4 mm apart, were placed in distilled water until completion of the cutting operation. Except where indicated otherwise, the sections used were adjacent to the apical crook (Figure 1).

Test solutions were maintained near pH 6.5 with 0.01 M phosphate buffer. Two per cent sucrose (w/v) was used except in tests of the effect of sucrose concentration upon longitudinal and lateral expansion. Ten ml of the test solutions were placed in each Petri dish 100 by 15 mm. Twelve to 20 (usually 15) randomized sections were placed in each dish, and, except where indicated otherwise, were incubated in the dark for 24 hours at $20 \pm 1^\circ\text{C}$. Diameters of a similarly randomized sample were measured immediately after cutting.

All sections were measured at $9\times$ magnification on the stage of a dissecting microscope with the aid of an ocular micrometer. Section length was measured to the nearest 0.1 mm, and diameters at the widest point were measured to the nearest

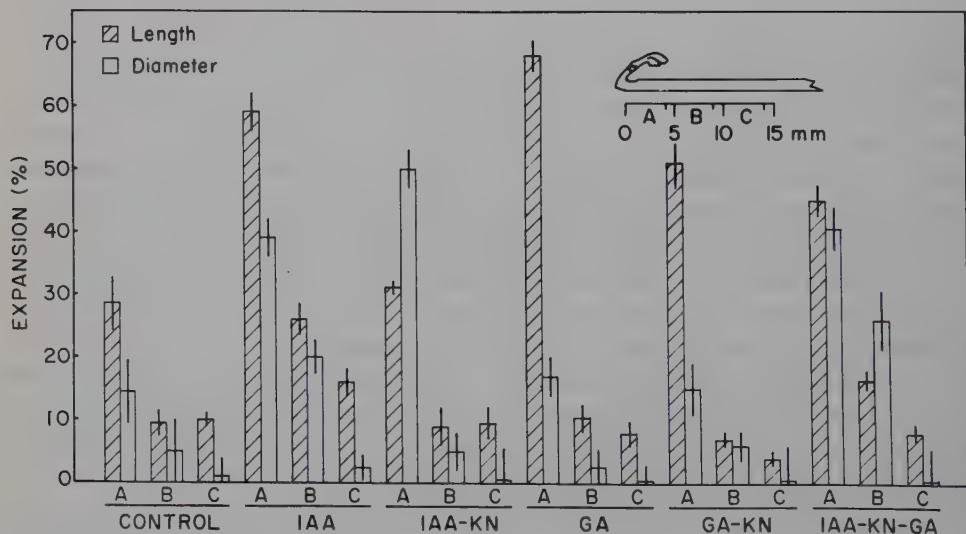


Figure 1. Effect of pea section location on longitudinal and lateral response in 24 hours to IAA, GA, IAA + KN, GA + KN, and GA + IAA + KN. Section location is indicated by the pea seedling sketch at upper right. All treatments, including controls, contained two per cent sucrose and 0.01 M phosphate buffer at pH 6.5. Growth substance concentrations were 1.0 $\mu\text{g/ml}$. Vertical lines represent three times the standard error.

0.02 mm. When the standard error, calculated for all measurements, exceeded five per cent, the data were discarded.

Microscopic tissue examinations were made on free-hand sections of fresh material mounted in water or lacto-phenol or on prepared slides. The latter were killed and fixed in Randolph's solution followed by the usual paraffin procedures. Safranin and fast green stains were used.

The KN used was obtained from the Sigma Chemical Company, and the IAA from the California Corporation for Biochemical Research. In all tests, GA was the potassium salt of gibberellic acid, obtained from the Nutritional Biochemicals Corporation. Stock solutions of these materials were prepared and stored in the dark near 0°C for not more than two weeks.

Results

Sections were cut to a uniform length of 4 mm., but initial diameter measurements were obviously variable. Measuring all section diameters at the time of cutting is time-consuming and involves risk of injury by handling or desiccation. Consequently, investigation was made of the variability of diameter measurements after seedlings were selected visually. These measurements indicated that the seedlings should be of about equal height, but of even more importance was the location of nodes. A node near the basal end of the section, or developing in the apical crook above, resulted in sections of comparatively large diameter. Therefore, only seedlings with three to four cm of internode tissue below the apical crook were selected.

To investigate the uniformity of diameters, sections were cut and measured on each of several days. The following average diameters and standard error values were obtained: 1.65 ± 0.02 , 1.68 ± 0.01 , 1.65 ± 0.01 , 1.73 ± 0.02 , 1.68 ± 0.02 , and 1.75 ± 0.02 mm. Thus, section diameters were quite uniform on any one day, though some day-to-day variability exists. Variation from one day to the next might reflect a difference in growing conditions, but more likely indicates a difference in selection. Consequently, diameter measurements of a randomized sample of 15 freshly cut sections were recorded at the start of each test.

The location of sections providing the greatest longitudinal and lateral expansion response in 24 hours to IAA, GA, KN, and sucrose was determined by selecting sections from three locations with reference to the apical crook. These were: 1) the four mm immediately below the apical crook, 2) from 5 to 9 mm, and 3) from 10 to 14 mm. below. Data in Figure 1 show that expansion in both longitudinal and lateral directions was greatest in the tissue immediately below the apical crook. The next-lowest sections responded to IAA but not to GA. The lowest sections responded a small amount or not at all.

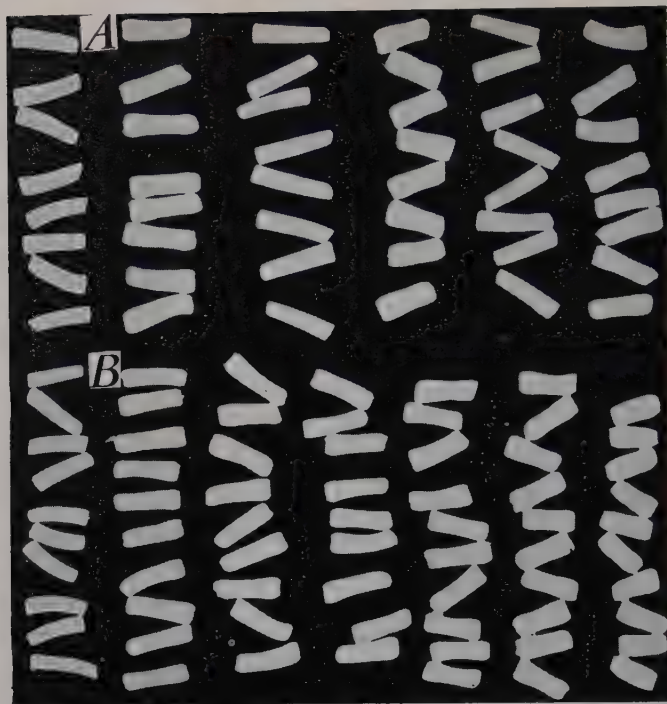


Figure 2. Response of 4-mm-long etiolated pea sections to growth substances after 24 hours (about 1.4 \times magnification). A. Response to GA, IAA, and KN, at concentrations of 1.0 $\mu\text{g}/\text{ml}$ in two per cent sucrose. Left to right: Sucrose control, IAA, GA, IAA + KN, GA + KN, and GA + IAA + KN. B. Response to various KN concentrations. Left to right: Sucrose control, 0, 0.001, 0.01, 0.1, 1.0, and 10 $\mu\text{g}/\text{ml}$ KN, all with 1.0 $\mu\text{g}/\text{ml}$ IAA except sucrose control.

With respect to the uppermost sections, sucrose alone and IAA with sucrose caused longitudinal expansion with a small lateral expansion. The addition of KN to the IAA and sucrose eliminated the longitudinal response nearly completely but stimulated a marked increase in diameter.

GA with sucrose stimulated a striking longitudinal response in pea sections, but with little or no lateral increase. The addition of KN decreased the GA-stimulated longitudinal expansion without materially stimulating lateral expansion. Addition of KN to combinations of IAA and GA resulted in sections exhibiting an intermediate response, *i.e.*, both longitudinal and lateral expansion. Thus, longitudinal expansion is increased when GA is added to IAA-KN combinations, whereas lateral expansion results when IAA is added to GA-KN combinations (Figure 2).

Histological comparisons of sections treated with IAA, GA, and IAA + KN, all with two per cent sucrose, revealed extensive stimulation of cell division in phloem parenchyma and rays after the latter treatment. In addition, after responding to IAA + KN, cortical cell diameters were larger, and lengths shorter, than similar sections with GA or IAA alone.

Expansion to near final length and diameter was completed within 24 hours. Another 24 hours resulted in only a small increase in length and

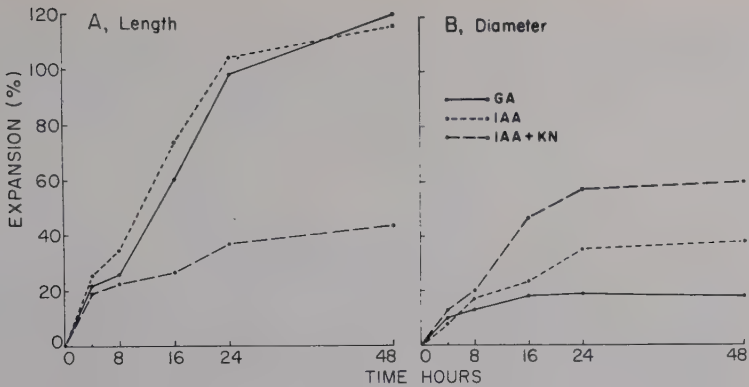


Figure 3. Longitudinal and lateral expansion of etiolated pea sections in response to GA, IAA, and IAA + KN after indicated periods. IAA and GA concentrations of 0.1 and KN concentrations of 1.0 $\mu\text{g/ml}$. were used, all with two per cent sucrose.

little or no added diameter expansion in response to GA, IAA, or IAA + KN (Figure 3).

The influence of sucrose on longitudinal and lateral expansion during GA and IAA treatments was studied in a series of experiments. Two per cent sucrose increased GA-induced elongation, with little or no increase in diameter. High sucrose concentrations strikingly reduced GA-stimulated longitudinal expansion, with little or no effect on diameter (Figure 4 A). With IAA, two per cent sucrose increased longitudinal expansion, with a modest lateral increase. With eight per cent sucrose, longitudinal expansion was inhibited while lateral expansion was stimulated in a manner similar to the lateral expansion with KN (Figure 4 B). The addition of KN to IAA with eight per cent sucrose resulted in a greater inhibition of elongation and an increased lateral expansion. However, the KN effect is maximum when it is added to IAA and sucrose concentrations that are near optimal for elongation, i.e., two per cent sucrose and 0.1 $\mu\text{g/ml}$ IAA (Figure 4 C).

When cobaltous chloride was added to a GA concentration of 0.1 $\mu\text{g/ml}$. in two per cent sucrose, longitudinal expansion was stimulated at concentrations of 4×10^{-5} M, and 8×10^{-5} M, but was inhibited at 16×10^{-5} M. Lateral expansion was not stimulated. A similar longitudinal stimulation, with little or no added lateral response, was noted when cobaltous chloride was added to IAA (0.1 $\mu\text{g/ml}$.) with two per cent sucrose. The addition of cobaltous chloride to IAA – KN combinations reduced the expected KN response. Lateral expansion was less and the length was greater because of a counteraction of the KN effect (Figure 5).

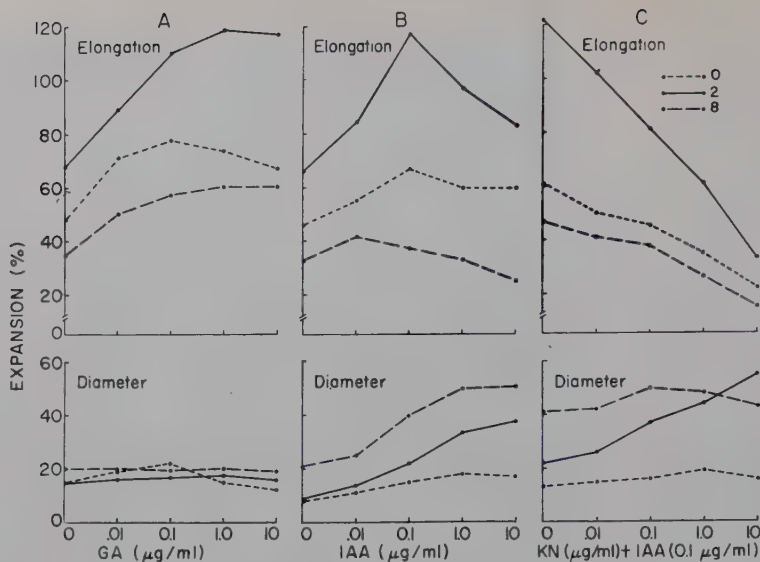


Figure 4. Longitudinal and lateral expansion response of pea sections in 24 hours to various concentrations of GA, IAA, and KN as influenced by sucrose concentration. Legend at upper right indicates per cent sucrose.

Without IAA, KN reduces longitudinal expansion but affects lateral expansion to only a very minor extent. Likewise, without sucrose, longitudinal inhibition is not accompanied by large lateral stimulus. When added to IAA (0.1 $\mu\text{g/ml.}$) in two per cent sucrose, KN concentrations as low as 0.001 $\mu\text{g/ml.}$ slightly inhibited the IAA-induced elongation. Though sections were

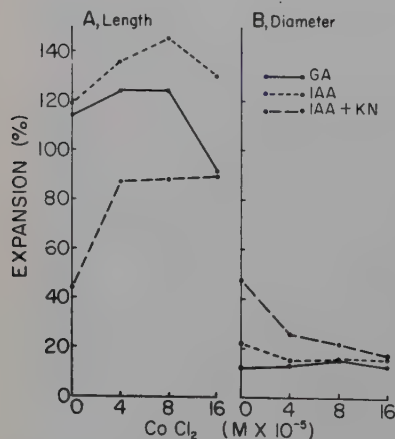


Figure 5. Effect of cobaltous chloride on longitudinal and lateral expansion response of pea sections in 24 hours to IAA (0.1 $\mu\text{g/ml.}$), GA (0.1 $\mu\text{g/ml.}$), and KN (1.0 $\mu\text{g/ml.}$).

shorter, the diameters were as large as the IAA controls, or larger. At KN concentrations of 0.1 $\mu\text{g/ml}$. or above, elongation is further inhibited and, in addition, stimulation of lateral expansion is readily detected (Figure 2 B). The fresh weight of sections responding to IAA + KN is less than with IAA alone.

Discussion

The striking lateral response of etiolated pea sections to combinations of KN with IAA, and the lack of a similar response to KN – GA combinations, illustrate a significant difference in the action of GA and IAA in cell expansion.

The lateral expansion caused by KN (with IAA) can possibly be explained by cell divisions in the radial direction. In addition, the extremely short cortical cells can result from cell divisions. The much enlarged diameters of these cells, however, cannot be explained so easily. Benzimidazole has been reported to cause a lateral expansion of pea sections without stimulating cell division, apparently by an effect on the geometry of growth (2). Similarly, ethylene causes longitudinal inhibition and lateral expansion of *Avena* coleoptiles without stimulation of cell division (7). Are the increased diameters of cortical cells here reported the result of a similar unidirectional expansion stimulation? Results of a histological study now under way will be reported.

The response by longitudinal inhibition and lateral expansion of the uppermost four-mm-long etiolated pea sections to KN (with IAA) appears to be as sensitive as their response by elongation to GA and IAA. Consequently, it appears that pea sections can be used to supplement other methods for studying materials in plant extracts that stimulate cell division.

The influence of large amounts of sucrose on lateral expansion necessitates the elimination of unknown amounts of sugars from plant extracts. However, sugars are nearly eliminated when plant extracts are reduced to dryness and then taken up in absolute ethanol before application to chromatographic paper. IAA and other indole auxins can be separated on chromatograms from materials providing a KN-like reaction. For example, chromatographic development with 70 per cent ethanol results in migration of the naturally occurring indole auxins to R_f 0.78 or above (14), whereas the kinin-like activity of *Taphrina* extracts is produced by materials at lower R_f values (15).

The extraction and chromatographic procedures used primarily to date with fungus cultures in liquid media are briefly summarized here. The plant material is extracted for about 24 hours near 0°C with sufficient 95

per cent ethanol to result in about a 70 per cent concentration in the culture solution. Solids are removed by centrifugation, and the liquid extract is reduced to dryness in a rotary "flash evaporator", avoiding temperatures above 35° C. A few ml. of cold absolute ethanol are added to the dry residue, and the resulting mixture is held overnight at about 0° C. After evaporation to about 1/2 ml., the substances soluble in absolute ethanol are applied with a tuberculin syringe to a line near the base of a 46-by-57-cm. sheet of Whatmann 3 MM chromatographic paper. After developing the unidirectional ascending chromatogram with 70 per cent ethanol for about 24 hours, it is dried and cut transversely into 10 strips of Rf 0.1 width each. Individual paper strips are placed in separate Petri dishes 100 by 15 mm. Paper strips above the solvent front are used for controls. To each Petri dish is added 10 ml. of a solution consisting of two per cent sucrose and 0.1 µg/ml IAA in 0.01 M phosphate buffer at pH 6.5. Fifteen pea sections are added to each dish and incubated for 24 hours in the dark at 20° C.

Concentrations of KN can likely be expressed best in terms of values that reflect both longitudinal inhibition and lateral expansion about equally. Used for this purpose was an arbitrary index number consisting of the sum of 0.4 times the longitudinal inhibition (mm.) plus the lateral stimulation (mm.). Since the original diameters are approximately 0.4 times the original length, only that fraction of the longitudinal inhibition is included in the index number. Otherwise, greater weight would be given to longitudinal inhibition than to lateral expansion. For example, the response to KN with two per cent sucrose and 1.0 µg/ml IAA (Figure 4 C) gave index numbers of 0, 0.3, 0.9, 1.3, and 2.0 for KN concentrations of 0, 0.01, 0.1, 1.0, and 10 µg/ml.

Summary

Etiolated pea sections responded to indoleacetic acid (IAA) and to gibberellin (GA) by elongation. The elongation was enhanced by addition of two per cent sucrose. Kinetin (KN) added to IAA with sucrose suppressed longitudinal expansion but increased lateral expansion. The addition of KN to GA in sucrose, on the other hand, suppressed longitudinal expansion with little or no stimulation of lateral expansion. Four-mm. sections adjacent to the apical crook were more responsive to both longitudinal and lateral expansion than lower or longer sections.

A large lateral expansion in response to KN required IAA and sucrose. The addition of cobaltous chloride, in contrast, permitted pea sections to undergo considerable elongation while inhibiting the KN-induced stimulation of lateral expansion.

The response of pea sections to KN may permit their use, in combination with paper chromatography, as a rapid bioassay for studies of substances that stimulate cell division in plant extracts.

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Effect of *in vitro* Preincubation with Cofactors on the Activity of the Indoleacetic Acid Oxidase of Peas

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Introduction

The indoleacetic acid (IAA) oxidase activity of pea homogenates is known to be enhanced by the manganous ion (Wagenknecht and Burris 1950, Hillman and Galston 1956), by substituted phenols (Goldacre, Galston, and Weintraub 1953, Hillman and Galston 1956), and by a natural cofactor found in the terminal buds of dark grown seedlings (Sharpensteen and Galston 1959). The enzyme is inhibited by a dialyzable substance produced in etiolated pea plumules some hours after exposure to red light (Hillman and Galston 1957) and found also in various parts of the light grown pea plants (Tang and Bonner 1948, Galston 1959). The effect of red light in promoting synthesis of the inhibitor is reversed by exposure of the tissue to far-red light immediately after the red light treatment. This and other evidence has led to the hypothesis that the inhibitor may be the controlling factor in the activity of the enzyme, which may in turn be involved in the control of growth and certain morphogenic phenomena.

The evidence presented below shows that the IAA oxidase activity of crude pea homogenates can be reversibly enhanced or inhibited by preincubation with cofactorial substances such as the manganous ion or 2,4-dichlorophenol (DCP). This observation has relevance for the reported induced formation of the enzyme (Galston and Dalberg 1954, Audus 1959), and for an understanding of the mechanism of the control of enzyme activity by enhancing

and inhibitory substances. Further, the recent isolation of the inhibitor from green pea leaves and the demonstration that it is a glucoside of quercetin furnishes new data for an understanding of the intermediary biochemistry of photomorphogenesis (Furuya, Galston, and Stowe 1961).

Material and Methods

Pisum sativum L. var. Alaska, seeds of which were obtained from Associated Seed Growers, Inc., New Haven, Connecticut, was used exclusively in this investigation. The seedlings were grown from seed planted in vermiculite and kept in an air conditioned dark room at $28^{\circ} \pm 1^{\circ}\text{C}$. Most of the experiments were conducted with crude homogenates of plumules of 7-day old peas; therefore, where red-light exposed peas were needed, 6-day old etiolated peas were treated with red light (approximately $1000 \text{ erg/cm}^2 \text{ sec.}$) for 30 minutes as described elsewhere (Hillman and Galston 1957).

Sixteen hours after the red light exposure, the terminal buds were harvested in the dark room under a dim green safe-light constructed by wrapping a 15 W Sylvania green fluorescent tube with 3 layers each of du Pont dark green and amber cellophane and a thickness of aluminum foil at each end. They were then kept in a deep-freeze for a short period of time, after which the crude homogenate was prepared as quickly as possible. Usually 1.2 g. of the frozen tissue was reduced to a powder with a chilled mortar and pestle and ground with cold 0.01 M phosphate buffer, pH 6.1, filtered through cellulose tissue to remove coarse debris, and finally made up to 50 ml. with the buffer. Two ml. of crude homogenate (24 mg. fresh weight/ml.) were immediately pipetted into each prepared preincubation mixture. Such a mixture consisted of 2 ml. of phosphate buffer (0.1 M, pH 6.1), supplemented by 1 ml. of MnCl_2 (10^{-3} M) and/or 1 ml. of DCP (10^{-3} M) and H_2O , in a final volume of 8 ml. The IAA destroying capacity was measured at the time of mixing (zero time) and after various periods of preincubation. The preincubation was carried out in an Aminco-Dubnoff metabolic shaking incubator (90 cycles/min.) at 30°C in darkness, except for certain experiments involving the effects of temperature or of illumination.

The IAA oxidase activity of the fresh and preincubated reaction mixture was assayed by the Salkowski colorimetric method (Tang and Bonner 1947, Hillman and Galston 1956). After various periods of preincubation of homogenate with cofactors, 2 ml. of IAA (10^{-3} M) were added to the reaction mixture, and the incubation continued. Then residual IAA was determined on a 1 ml. aliquot at various intervals after the addition of IAA. In this assay, the concentration of each constituent was as follows; IAA, $2 \times 10^{-4} \text{ M}$; MnCl_2 , 10^{-4} M ; DCP, 10^{-4} M ; phosphate buffer, 0.02 M.

Results

1. Crude homogenate of plumules pretreated with red light

All experiments described in this section concern the crude homogenate of terminal buds of plants exposed to red light 16 hours prior to harvest. When the IAA oxidase activity of such crude homogenates is determined in

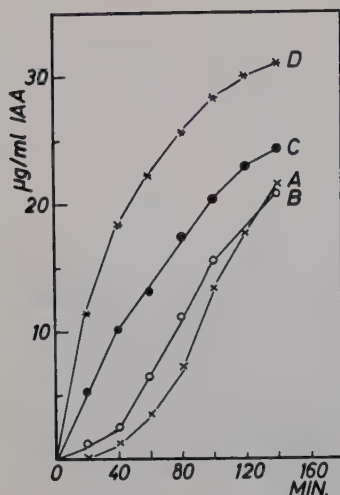


Figure 1. Kinetics of IAA destruction by a crude homogenate from red light pretreated pea plumules, measured at various periods of time after preincubation with Mn^{++} (A, 0 min.; B, 60 min.; C, 120 min.; D, 160 min. after preincubation, respectively). The final reaction mixture consists of 2 ml. of IAA (10^{-3} M), 2 ml. of homogenate (24 mg.f.w./ml.), 1 ml. of $MnCl_2$ (10^{-3} M), 2 ml. of phosphate buffer (1/10 M, pH 6.1), and 3 ml. of H_2O . On the ordinate µg/ml. IAA destroyed.

the presence of Mn^{++} added together with the IAA substrate, the kinetic curve showed a long lag period. However, if the activity is determined after a period of preincubation with Mn^{++} , the lag period is progressively diminished and the rate of destruction of IAA accordingly increased (Figure 1). Thus, if the activity at some fixed time after addition of IAA is plotted against the time of preincubation with Mn^{++} , it is seen that the longer the period of preincubation, the higher is the rate of IAA destruction (Figure 2). The final maximum activity is essentially equal to that of an inhibitor-free homogenate derived from totally etiolated plumules. We thus believe that this activation by Mn^{++} represents a progressive disappearance of the inhibitor causing the lag period.

Figure 2. The effect of preincubation with Mn^{++} on the IAA destroying capacity of crude homogenate of red light exposed plumules. The reaction mixture is same as in Figure 1. On the abscissa preincubation time, on the ordinate µg/ml. IAA destroyed in 40 min.

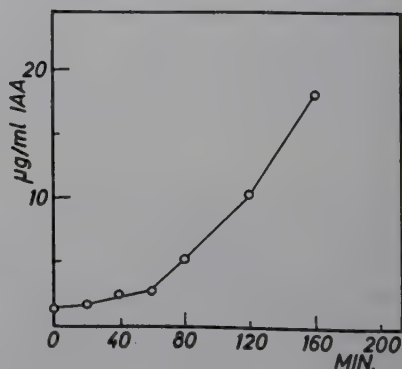
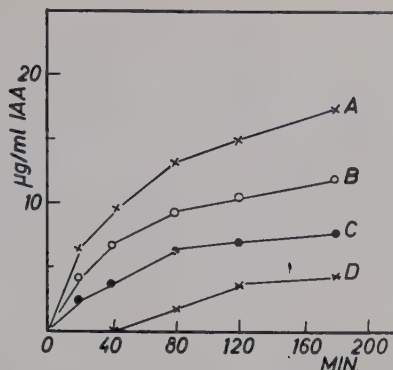


Figure 3. Kinetics of IAA destruction by a crude homogenate from red light pretreated pea plumules, assayed at various periods of time after preincubation with DCP (A, 0 min.; B, 20 min.; C, 40 min.; D, 120 min.). The final reaction mixture consists of 2 ml. of IAA (10^{-3} M), 2 ml. of homogenate (24 mg.f.w./ml.), 1 ml. of DCP (10^{-3} M), 2 ml. of phosphate buffer (1/10, pH 6.1), and 3 ml. of H_2O . On the ordinate IAA destroyed.



On the other hand, if DCP is added to the reaction mixture instead of Mn^{++} , no lag period can be detected and high activity is immediately observed. However, with increasing periods of preincubation with DCP, the IAA destruction activity of the homogenate is progressively decreased (Figure 3). If the activity is plotted against the period of time of preincubation with DCP, it is clear that the longer the preincubation, the lower is the rate of IAA destruction, until complete inhibition results (Figure 4). If fresh DCP is added to the DCP-preincubated reaction mixture, the activity is not affected

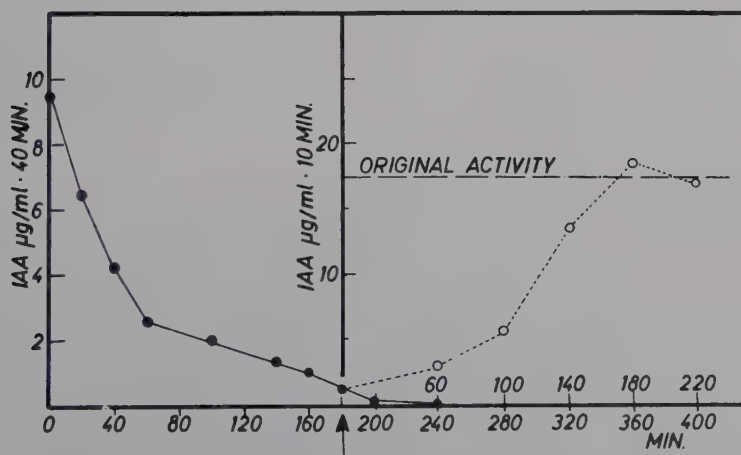


Figure 4. The IAA oxidase activity of a crude homogenate of red light pretreated plumules preincubated with DCP (●—●—), and subsequently with Mn^{++} (○ ○) which was added at the arrow. In the assay of the latter, the activity was measured in the presence of both DCP and Mn^{++} as the result of successive incubation. On the abscissa preincubation time, on the ordinate IAA destroyed.

Table 1. IAA oxidase activity of DCP-treated homogenates with or without freshly added DCP. Constituents of the initial reaction mixture; 2 ml. of crude homogenate from red light pretreated plumules (24 mg.f.w./ml), 1 ml. of DCP (10^{-3} M), 2 ml. of phosphate buffer (1/10 M, pH 6.1), and 2 ml. of H_2O . Then, 1 ml. of DCP (10^{-3} M) or H_2O was added, and after subsequent preincubation, 2 ml. of IAA (10^{-3} M) was pipetted to this reaction mixture.

Duration of preincubation (minutes)	IAA oxidase activity (μ g/ml IAA destroyed in 40 min.)	
	Added DCP	Added H_2O
0	6.3 ¹	8.3
60	1.6	1.6
120	0.8	0.8
180	0.2	0.0

¹ This lowered activity, due to supraoptimal concentration of DCP.

(Table 1), indicating that the loss of activity is not due to a disappearance of DCP needed as a cofactor, but probably to a conversion of DCP to an inhibitory substance. When Mn^{++} is added to a reaction mixture completely inhibited by prolonged preincubation with DCP, the activity gradually increases and finally returns to the original uninhibited value (Figure 4, right).

If the homogenate is preincubated with both Mn^{++} and DCP, the data of Figure 5 are obtained. The enzyme shows high initial activity, followed by the inhibition due to preincubation with DCP and later by the activating effect of Mn^{++} . Plumular homogenates from red light pretreated plants preincubated in the absence of Mn^{++} and DCP showed extremely low activity

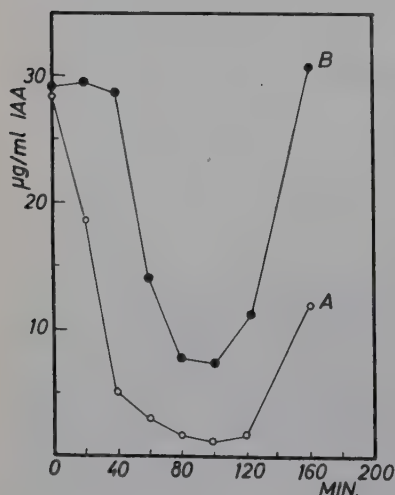


Figure 5. The effect of preincubation with both Mn^{++} and DCP on the IAA destroying capacity of crude homogenate of red light pretreated plumules. A (○—○) presents the activity as μ g/ml. IAA destroyed in 20 min. after various period of preincubation, and B (●—●) presents that in 40 min. The final reaction mixture consists of 2 ml. of IAA (10^{-3} M), 2 ml. of homogenate (24 mg.f.w./ml.), 1 ml. of $MnCl_2$ (10^{-3} M), 1 ml. of DCP (10^{-3} M), 2 ml. of phosphate buffer (1/10, pH 6.1), and 2 ml. of H_2O . On the abscissa preincubation time, on the ordinate IAA destroyed.

(due to the presence of the red light-induced inhibitor) which remained constant.

To summarize, (1) preincubation of crude homogenates from red light exposed plumules with Mn^{++} progressively increases IAA-destroying capacity, (2) preincubation with DCP progressively decreases this activity, and (3) preincubation with both Mn^{++} and DCP results first in the inhibition due to DCP preincubation and later in the reactivation by Mn^{++} , (4) both the Mn^{++} and DCP effects are markedly temperature dependent (Figures 7 to 9).

2. Crude homogenate of dark grown plumules

Crude homogenates of etiolated plumules are known to contain relatively large quantities of a dialyzable natural cofactor of IAA oxidase (Sharpsteen and Galston 1959), and can destroy IAA without any addendum such as Mn^{++} or DCP. Such homogenates were studied to see whether some shift of the IAA oxidase activity would occur as a result of preincubation. The activity of such a preparation gradually decreases on preincubation with no experimentally added cofactors (Figure 6; A). If the activity were determined after a period of preincubation with Mn^{++} , the IAA destroying capacity at first gradually rose, then slowly decreased (Figure 6; B). When the homogenate was incubated with DCP instead of Mn^{++} , the activity was initially twice as high (Figure 6; C), but declined markedly with successively longer preincubation with DCP, exactly like homogenates of red light pretreated plumules. If the activity is plotted against the time of preincubation with DCP, it is found that the longer the period of preincubation, the lower is the rate of IAA destruction, until the activity becomes lower than

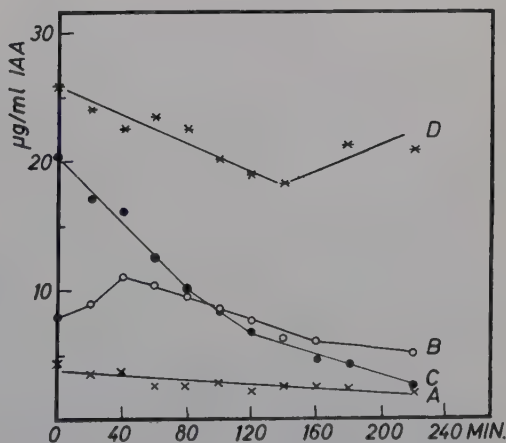


Figure 6. The IAA oxidase activity of crude homogenate from dark grown plumules, after various period of preincubation with no addendum (A) with Mn^{++} (B) with DCP (C) and with both Mn^{++} and DCP (D). A, B, C assayed after 40 min. of IAA oxidation, D after 10 min. On the abscissa preincubation time, on the ordinate IAA destroyed.

Table 2. *The effect of preincubation on the IAA oxidase activity of a synthetic system, consisting of 2 ml. of crude homogenate from dark grown plumules (24 mg.f.w./ml), 1 ml. of natural inhibitor from green pea leaves (Galston 1959), 2 ml. of phosphate buffer (1/10 M, pH 6.1), and with or without 1 ml. of $MnCl_2$ (10^{-3} M) and/or of DCP (10^{-3} M), and H_2O ; the final volume is 8 ml.*

Period of time of preincubation (minutes)	IAA oxidase activity in the presence of DCP	
	$\mu g/ml$ IAA destroyed in 40 min.	$\mu g/ml$ IAA destroyed in 10 min.
0	17.1	23.1
60	8.6	21.6
120	7.0	17.5
180	4.0	12.3
240	0.0	18.4

those preincubated with Mn^{++} for the same period of time. It is also evident that, if Mn^{++} is added to the DCP-treated reaction mixture during the preincubation, the inhibition induced by DCP is gradually removed, while if fresh DCP is added to the DCP-pretreated reaction mixture the activity is not affected at all. This behavior is the same as those with red light exposed plumules.

When the crude homogenate prepared from dark grown plumules is supplemented by the natural inhibitor obtained from green pea leaves (Galston 1959), and the mixture preincubated with cofactors, the IAA destroying capacity is altered in a way similar to that of red light pretreated plumules (Table 2). This indicates a chemical similarity and possibly identity of the natural inhibitor from green peas with that from red light exposed etiolated plumules.

3. Factors controlling preincubation effects

a) *Temperature.* — The rate of change of IAA oxidase activity by preincubation is dependent upon temperature. However, the direction of the shift of activity is determined by the cofactor added, and is not changed by temperature.

With respect to preincubation with Mn^{++} , no shift of activity was detectable at $2^\circ C$ (Figure 7, $\square-\square-$), but preincubation temperatures of $20^\circ C$, $30^\circ C$, and $40^\circ C$ yielded increasingly rapid rates of shift of activity. Some thermal inactivation of the enzyme began to appear after 2 hours of preincubation at $40^\circ C$.

The preincubation effects with DCP and with Mn^{++} and DCP were also markedly temperature dependent (Figures 8 and 9).

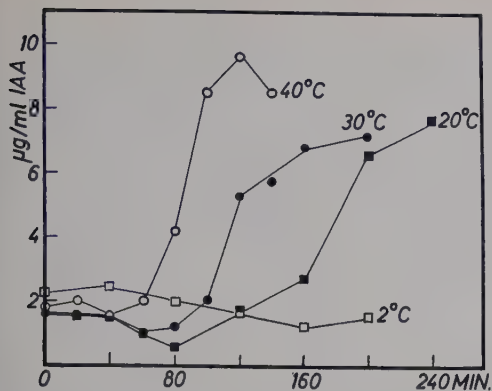


Fig. 7.

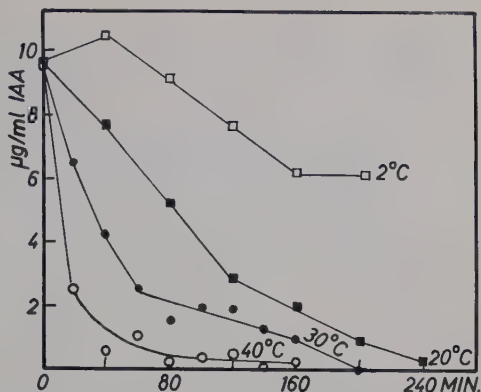


Fig. 8.

Figure 7. *Effect of temperature on preincubation of crude homogenate from red light pretreated plumules with Mn^{++} ; at 2, 20, 30, and 40°C. The constituents of the reaction mixture were same as those in Figure 1. After preincubation at different temperatures, IAA oxidase activity was measured at 30°C as usual. On the abscissa preincubation time, on the ordinate IAA destroyed in 40 min.*

Figure 8. *The effect of temperature on preincubation of crude homogenate from red light pretreated plumules with DCP; at 2, 20, 30, and 40°C. The constituents of the reaction mixture were same as those in Figure 3. After preincubation at different temperatures, IAA oxidase activity was measured at 30°C. On the abscissa preincubation time, on the ordinate IAA destroyed in 40 min.*

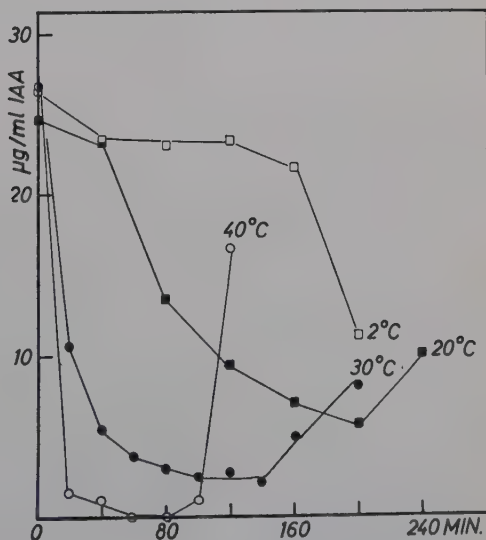


Figure 9. *Effect of temperature on preincubation of crude homogenate from red light pretreated plumules with both Mn^{++} and DCP at 2, 20, 30, and 40°C. The constituents of the reaction mixture were same as those in Figure 5. After preincubation at different temperatures, IAA oxidase activity was measured at 30°C. On the abscissa preincubation time, on the ordinate IAA destroyed in 10 min.*

Table 3. *Effect of light upon preincubation with Mn^{++} and DCP on IAA oxidase activity of the crude homogenate from red light pretreated plumules.*¹ Preincubation was carried out in the dark room, where a green safe lamp was turned on only when needed. ² Illuminated by an incandescent lamp (100 W, 115 V) 25 cm above the shaking incubator.

Condition of preincubation			IAA oxidase activity (μ g IAA destroyed/reaction mixture)	
Cofactor added	Temperature (°C)	Illumination	Measured after minutes of preincubation	
			40	120
Mn^{++}	20	dark ¹	1.5	1.8
		light ²	1.5	2.5
	40	dark	1.4	10.0
		light	1.8	10.5
DCP	20	dark	9.3	4.4
		light	8.8	5.2
	40	dark	0.6	0.5
		light	0.8	0.6
Mn^{++} & DCP	40	dark	0.2	8.8
		light	0.4	6.9

b) *Light*. — The preincubation effects in darkness and in light were compared, using a crude homogenate from red light pretreated plumules. The results, summarized in the Table 3, show that the preincubation effect is not influenced by light.

c) *Oxygen*. — Preincubation treatments under air and under commercial nitrogen were compared. Little change in IAA oxidase activity was observed under a nitrogen atmosphere after a period of preincubation with Mn^{++} , DCP, or neither (Table 4). The preincubation effects described above therefore seem to require oxygen.

Table 4. *Preincubation of crude homogenate from red light pretreated plumules under aerobic and anaerobic conditions (Commercial nitrogen gas).*

Cofactor added		IAA oxidase activity (Salkowski color decrease/40 min.) Preincubation period (minutes)		
		0	60	120
None	air	5	2	0
	N ₂	5	4	5
Mn^{++}	air	32	38	51
	N ₂	32	36	36
DCP	air	55	14	2
	N ₂	55	51	49

Table 5. *Preincubation effect of various metallic ions on IAA oxidase activity of crude homogenate from red light pretreated plumules.* Preincubation reaction mixture consists of 2 ml. of homogenate, 2 ml. of phosphate buffer, 1 ml. of metallic ion (10^{-3} M), and 3 ml. of H_2O .

Ion added	series of experiment	IAA oxidase activity (Δ Salkowski color/120 min) Period of preincubation (minutes)		
		0	60	120
None	I ¹	40	32	27
	II ¹	34	18	8
CaCl ₂	I	42	22	14
	II	36	20	12
CeCl ₃	I	52	41	33
	II	34	36	10
CoCl ₂	I	35	32	19
	II	34	16	6
CuCl	I	32	37	15
	II	36	16	8
FeCl ₂	I	40	58	47
	II	34	26	20
MgCl ₂	I	33	34	23
	II	30	26	14
MnCl ₂	I	73	74	86
	II	104	111	162
NiCl ₂	I	40	27	21
	II	30	18	4
ZnCl ₂	I	25	21	15
	II	30	16	4

¹ Amount of the red light induced inhibitor or its precursor in tissue varies in each experiment even under the same physical treatment.

d) *Metallic ions.* — An attempt was made to substitute other metallic ions for Mn^{++} in this system. It is clear from the data of Table 5 that preincubation with Ca^{++} , Ce^{+++} , Co^{++} , Cu^+ , Fe^{++} , Mg^{++} , Ni^{++} , or Zn^{++} was ineffective. It therefore seems that Mn^{++} is specific in its activation of the IAA destroying capacity of homogenates from red light exposed plumules.

4. Possible mechanism of the preincubation effect of crude homogenate

The above experiments show that, when a crude homogenate is used as a source of IAA oxidase, the IAA destroying capacity varies over a wide range, depending on the pretreatment conditions. However, if dialysed homogenate is employed as an enzyme source, IAA oxidase activity is constant even after a period of preincubation with Mn^{++} or DCP. In these experiments,

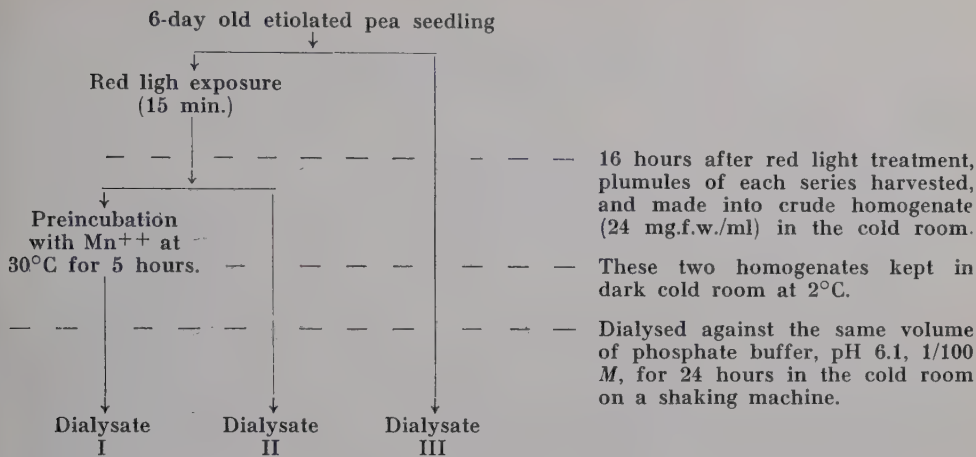
Table 6. *The comparison of preincubation effect between crude and dialysed homogenate of red light pretreated plumules.*

Preincubated with	Enzyme used	IAA oxidase activity (μg IAA destroyed/80 min.) Period of time of preincubation (minutes)			
		0	60	120	180
DCP	Crude dialysed	14.5	5.2	2.2	0
		4.0	4.0	4.0	4.1
Mn^{++}	Crude dialysed	8.2	13.0	20.1	26.5
		1.9	1.0	1.3	2.0

the crude homogenate from red light pretreated plumules was prepared and 20-ml. portions were dialysed overnight in cellophane membranes against 5 l. of 0.01 *M* phosphate buffer, pH 6.1, at about 2°C, followed by the preincubation with Mn^{++} or DCP at 30°C for 0, 60, 120, and 180 minutes respectively. The IAA oxidase activity of each sample was then measured. The results are presented in Table 6. The same results were found in experiments with a dialysed homogenate from dark grown plumules. The preincubation of the inhibitory dialysate with Mn^{++} does not produce any activation in the absence of enzyme. These experiments show that the effect of preincubation, while enzymatically mediated, is not on the enzyme itself but rather on dialyzable substance(s) present in the crude homogenate.

In support of this hypothesis, spectrophotometric evidence has been obtained which shows that a natural constituent of plumule tissue, increases in amount after red light exposure, and disappears from the homogenate after preincubation with Mn^{++} . The ultraviolet absorption peaks of this unknown compound (267 and 316 $\text{m}\mu$) are identical with those of a naturally occurring inhibitor of IAA oxidase of peas which has just been isolated in crystalline form and partially characterized as a previously undescribed flavanol complex (Furuya, Galston, and Stowe 1961). The experimental evidence is as follows.

Three kinds of dialysate of pea homogenates were prepared by the procedures described in Table 7, and the ultraviolet spectrum of each dialysate measured against one of the other dialysates, in various combinations. It was found that a substance having UV absorption maxima at 267 and 316 $\text{m}\mu$ was present to a much greater extent in the dialysate of red light pretreated sample than in that of the dark grown one (Figure 10, b). This substance completely disappeared after incubation with Mn^{++} (Figure 10, a and c). The natural IAA oxidase inhibitor, produced in etiolated pea plumules after red light exposure, has UV absorption maxima at 267 and 316 $\text{m}\mu$ in acidic

Table 7. *The procedure for the preparation of three kinds of dialysates.*

or neutral solution (Figure 10, d), and yields a flavanol, a phenylpropanoid, and glucose upon hydrolysis. It therefore appears that the preincubation effect with Mn^{++} is due to destruction of this flavonol complex of peas.

The basic finding of this paper are summarized in Figure 11. Earlier workers have already found that dialysis of a homogenate of pea epicotyls

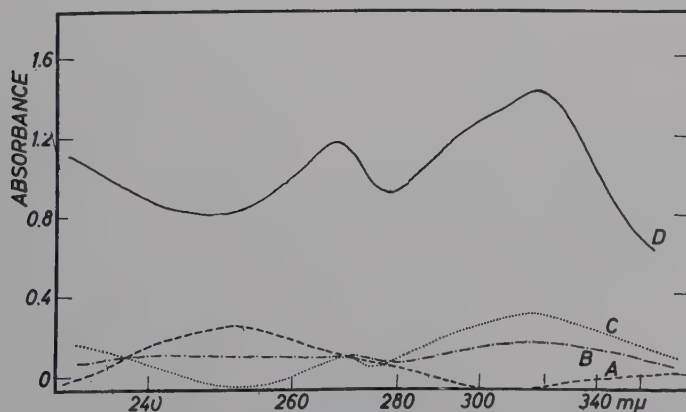
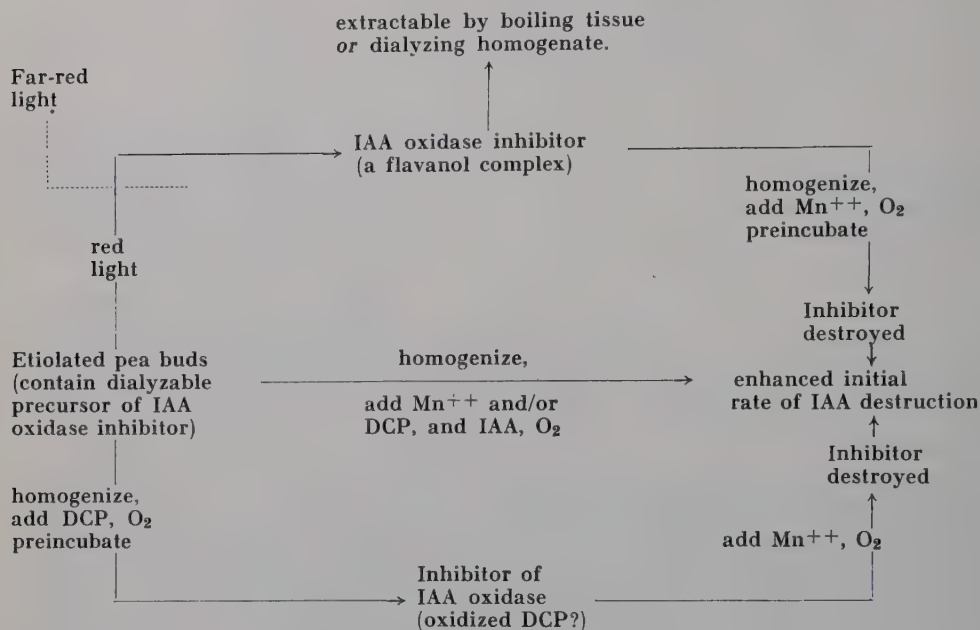


Figure 10. *The ultraviolet absorption spectra of the dialysates (24 mg. f.w. equivalent/ml.), which were obtained by the procedure in Table 7, and of the naturally occurring inhibitor of IAA oxidase from peas (d). A, measured the dialysate I against dialysate III; B, dialysate II against dialysate III; C, dialysate II against dialysate I; D, a flavanol complex obtained from pea plumules exposed to red light (ethanolic solution). On the abscissa wave length, on the ordinate absorbance.*

Figure 11. A summary of the conditions controlling the level of IAA oxidase inhibitor in pea buds and homogenates.



grown in weak red light led to a substantial increase in IAA oxidase activity, owing to the removal of a naturally occurring inhibitor (Tang and Bonner 1948, Galston and Baker 1951), and that about one-third of the activity of crude whole cytoplasm of bean roots or etiolated pea epicotyls is lost upon dialysis (Wagenknecht and Burris 1950), due probably to the removal of a natural cofactor. Further, an inhibitor inductively produced *in vivo* by red light (Hillman and Galston 1957) and a natural cofactor (Sharpensteen and Galston 1959) have been reported in pea plumules, the latter occurring in highest quantities in totally etiolated tissue.

Discussion

The manganous ion and DCP, both known cofactors of pea IAA oxidase, show completely different effects when preincubated with crude pea homogenate; Mn⁺⁺ seems to be involved in the disappearance of inhibitor, while DCP, despite its activating effect when added together with IAA, seems to be involved in the production of inhibitor. At least two different systems

appear to be concerned with these preincubation effects, and we shall discuss them separately.

The effect of Mn^{++} in preincubation was first found in kinetic studies in the disappearance of the lag period, and this is now thought to be due to the degradation of the natural inhibitor. However, many other reasons have been advanced to explain the often-noted lag periods in IAA oxidation (5, 13, 17, 22). For instance, the lag period for *in vitro* IAA decarboxylation and oxidation has been considered to represent the time necessary for the system to build up a threshold concentration of *manganic* ion (Maclachlan and Waygood 1956), or free radical (Yamazaki and Souzu 1960). On the other hand, Galston and Dalberg (1954) found that "in vivo" IAA destruction by young tissue of etiolated pea seedlings occurs only after a lag period of 10–20 minutes, which they considered was the time required for the induced formation of the oxidase in response to the high concentration of exogenous auxin. Further, Sharpsteen and Galston (1959) have found that at sub-optimal concentration of Mn^{++} there is a pronounced lag period which falls off rapidly to a negligible level, the concentration at which this level is reached being approximately optimal. These four theories seem not to be adequate to explain the preincubation effects with Mn^{++} , which is best interpreted in terms of destruction of inhibitor. Recently Sondheimer and Griffin (1960) have reported that aqueous extracts of acetone precipitates from etiolated pea epicotyls contain heat-stable substances which decrease the inhibition produced by chlorogenic acid.

It would be interesting to know whether the Kenten and Mann (1949, 1950) system is involved in the preincubation effect of pea homogenate with Mn^{++} . The natural pea inhibitor seems to be very complicated as a substrate; recent evidence (Furuya, Galston, and Stowe 1961) shows that, when the inhibitor is destroyed by a peroxidase- H_2O_2 system, the peak at 316 m μ (see Figure 10; d) declines while the peak at 267 m μ remains intact, and that, when the inhibitor is destroyed by the Mn^{++} -IAA oxidase system, the peak at 267 m μ disappears but little change takes place at 316 m μ .

Mudd and Burris (1960) reported that Mn^{++} can be replaced by Ce^{+++} in the peroxidase-catalysed oxidations of IAA, but the same is not true in the preincubation effect in pea homogenates.

No crucial evidence has been obtained with respect to the mechanism of the preincubation effect with DCP. Two possible situations might be expected; (a) in the presence of DCP, a precursor of natural inhibitor found in plumule tissue is converted to the active form of inhibitor, or (b) DCP itself is oxidized by preincubation to produce a dihydroxy phenol, known to be inhibitory to IAA oxidase *in vitro* (Goldacre *et al.* 1953). The second possibility seems to be more likely, because of the fact that the preincuba-

tion with DCP did not increase the amount of natural inhibitor as determined spectrophotometrically.

As already discussed elsewhere (Hillman and Galston 1957, Galston 1959), the naturally occurring inhibitor of IAA oxidase of peas might be physiologically important as a substance relating auxin metabolism to photomorphogenesis. Alternatively, it may merely be an indicator of a basic control of flavonoid biosynthesis by the photomorphogenic system, as already proposed by Siegelman and Hendricks (1958).

Summary

Previous work has shown that a diffusible inhibitor of indoleacetic acid oxidase is produced in the terminal buds of etiolated peas previously exposed to morphogenically active red light. Preincubation of homogenates of such tissue with manganous ion progressively increases IAA destroying capacity, while preincubation with 2,4-dichlorophenol (DCP) decreases this activity. When Mn^{++} and DCP are both present in the preincubation mixture, the inhibition due to DCP is manifested at first, but is later overcome by Mn^{++} effect. Preincubation of crude homogenates from dark grown plumules with DCP also results in inhibition of subsequent IAA destruction. Mn^{++} also reverses this inhibition, but has little effect on an inhibitor-free system from completely etiolated peas.

The inhibition induced by red light exposure, by preincubation with DCP, or by the addition of inhibitor extracted from green pea leaves, cannot be reversed by further addition of DCP, but can be reversed by subsequent incubation with Mn^{++} . Thus Mn^{++} appears to activate the enzyme complex by causing a disappearance of inhibitor.

The preincubation effects are strongly affected by temperature and oxygen, but are not influenced by light. They are also dependent on dialyzable factors present in the crude homogenate, for when dialyzed homogenate was used instead of the crude, no effect of preincubation was evident in the subsequent IAA oxidase activity assay. Other metallic ions such as Ca^{++} , Ce^{+++} , Co^{++} , Cu^{+} , Fe^{++} , Mg^{++} , Ni^{++} , and Zn^{++} could not be substituted for Mn^{++} .

The natural inhibitor has recently been isolated in crystalline form and partially characterized as a flavonol complex. The effect of Mn^{++} and of DCP are being further studied in the light of this new knowledge.

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The Metabolism of 3-Amino-1,2,4-Triazole by Canada Thistle and Field Bindweed and the Possible Relation to its Herbicidal Action

By

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Introduction

A difference in sensitivity to 3-amino-1,2,4-triazole (amitrole) between a relatively susceptible plant (Canada thistle, *Cirsium arvense* (L.) Scop.) and resistant field bindweed (*Convolvulus arvensis*) has been reported (6). One reason for this difference in sensitivity was penetration. After the droplets containing amitrole applied to these plants had dried, penetration into thistle was substantially better than penetration into bindweed and was considered to be a partial explanation for the difference in sensitivity between the two species (6).

It has also been postulated that differences in the metabolism of amitrole might also account for differences in sensitivity between these two species (6). Evidence to substantiate that a difference in the metabolism of amitrole exists between Canada thistle and field bindweed is presented. Also, consideration is given to the metabolism of amitrole with respect to its translocation and herbicidal activity.

Materials and Methods

The methods used to grow plants have been described (6). The procedure used to obtain samples for analyses of amitrole was a modification of methods used by other workers (1, 9, 11, 13). Essentially, this involved thoroughly washing the leaves to which amitrole had been applied with water to remove any residual

amitrole. The plants were then removed from the soil, washed again, and ground in a Waring blender with approximately 2 vol water. The homogenate was heated at 70° for 5 min. The insoluble fraction was removed by filtration and the filtrate was passed through a cation-ion exchange resin (Amberlite IR-120, H-form, 225 g. wet wt) in a 50-ml. burette. The resin was washed thoroughly with water and then the amitrole was eluted with NH_4OH (3 N, 600 ml.). The NH_4OH eluate was concentrated on a hot plate to near dryness, made up to a standard volume, and analyzed for amitrole according to the method previously described (6).

Free reducing sugars were determined by extracting fresh tissue with 80 % boiling ethanol for 2 hr. The insoluble materials were removed by filtration and the ethanol then removed by distillation. Water was added to the alcohol-free residue and this was passed through a mono bed resin (Amberlite MB-1). The eluate from the resin was made to volume and tested for free reducing sugars according to the procedure of Umbreit *et al.* (14).

Catalase activity was determined by the manometric method described by Landon (8). Briefly, plants were sampled by taking leaves of the same age and, after removing the midrib and large veins, comminuting known weights with equal amounts of precipitated chalk, a small amount of sand and a little distilled water in a mortar. After grinding was completed, water was added to make a final dilution of one part of tissue to 50 parts of water. The tissue preparation (2 ml.) was placed in one arm of a Y-tube and hydrogen peroxide (2 ml., 0.068 M) was placed in the other arm. The tube was equilibrated at 300 and then agitated by a reciprocating motion that moved its contents from arm to arm 40 times per minute during the period of measurement (10 min.). All measurements were made in duplicate.

Early workers have reported at least two metabolic derivatives of amitrole by a radioisotopic method (12) and by a colorimetric method (11). Recent workers (4, 5) have shown as many as 13 compounds are derived from C^{14} -labeled amitrole. To determine if metabolites of amitrole are produced in thistle plants, amitrole (500 $\mu\text{g/day}$) was applied to a different leaf of greenhouse-grown plants 7 consecutive days. Nine days after the first treatment, the plants (ca. 200 g. fresh wt of stem and leaf tissue for each plant) were harvested individually. Following concentration of the NH_4OH eluate from the cation resin, an aliquot was chromatographed one-dimensionally in isopropyl alcohol-ammonia-water (6:2:2) on Whatman No. 1 filter paper. The chromatogram was sprayed with the following three solutions in order: 1 % NaNO_2 in water; 20 % conc HCl in water; and 0.25 % recrystallized H-acid (8-amino-1-naphthol-3,6-disulfonic acid) dissolved in 50 % acetone-water. The extract was chromatographed in a different solvent system, *n*-butanol-ethanol-water (52.5:32.0:15.5) and a section was cut from the origin to a point corresponding to an R_f of 0.1. This section (0.0—0.1) was eluted with water, concentrated, and rechromatographed in the ammoniacal isopropanol solvent. Additional details of the methods used for specific experiments will be given in the Results.

Results

Chromatographic separation of the Canada thistle preparation in isopropyl alcohol-ammonia-water resulted in the location of three pink to orange-pink spots which appeared immediately after spraying with the H-acid. R_f values for these spots are given in Table 1.

Table 1. The *R_f* value of three compounds from the Canada thistle extract and the value for pure amitrole in two solvent systems.

Solvent system	<i>R_f</i>			
	Amitrole	A	B	C
Isopropyl alcohol-ammonia-water (6:2:2).....	0.88	0.87	0.74	0.66
<i>n</i> -Butanol-ethanol-water (52.5:32.0:15.5)	0.59	0.58	0.17	0.07

R_f values for the second solvent system used (*n*-butanol-ethanol-water) are also given in Table 1). After rechromatographing the 0.0–0.1 zone, a single spot was found having a *R_f* of 0.67. Thus, both “C” compounds (Table 1) having the lowest *R_f* in both solvent systems are probably the same. The three areas having a positive reaction with H-acid were arbitrarily named in order of their descending *R_f* values: amitrole, unknown I, and unknown II, corresponding to “A”, “B”, and “C”, respectively (Table 1).

The same results were obtained when the above procedure was qualitatively repeated with bindweed. Both unknowns I and II appeared to be chromatographically identical with the two unknowns obtained from thistle.

To determine if all the amitrole which entered the plant was converted into unknowns I and II, amitrole (3.0 mg) was applied in six 500- μ g treatments over a 2-day period to individual thistle plants on three different leaves. After washing and removing the treated leaves, the plants were individually harvested and analyzed chromatographically for amitrole. Amitrole could be detected in each plant up to 20 days following application. The plants were dying 20 days after amitrole application and no further harvests were made.

When this experiment was repeated with single bindweed plants, no amitrole could be detected, so a larger population was employed. Amitrole (8.25 mg.) was applied in 250- μ g. treatments (750 μ g/plant) over a 24-hr period to 11 bindweed plants. After 90 hr., all plants were combined into one group and harvested as described for the thistles. Both unknowns I and II were present but no amitrole could be detected in the composite of 11 plants 90 hr. following application.

Pyfrom *et al.* (10) observed that amitrole, when present, caused a reduction in catalase activity. The above chemical analysis indicated amitrole was absent in bindweed whereas it could be detected in thistle. Thus, from the conclusion of Pyfrom *et al.* (10) catalase activity of thistle should be suppressed while the catalase activity of treated bindweed should be normal. To test this, amitrole (250 μ g) was applied to three different thistle and bindweed plants. Ten days following application discolored leaves of treated plants were removed and individually analyzed for catalase activity. Corresponding

Table 2. *Catalase activity of discolored thistle and bindweed leaves from plants treated with amitrole (250 μ g) and normal leaves of untreated plants (averages and standard deviations of three different plants).*

Treatment	Thistle		Bindweed	
	Catalase activity (O ₂ ml/10 min.)	Activity %	Catalase activity (O ₂ ml/10 min.)	Activity %
No amitrole	20.5 \pm 0.37	100	13.7 \pm 0.85	100
Amitrole	12.7 \pm 0.96	62	13.8 \pm 1.15	101

leaves of three untreated plants were also removed and analyzed as the controls (Table 2). Definite suppression of catalase activity was observed in thistle whereas no reduction in activity was found in bindweed.

Racuson (11) observed the production of two metabolites when excised bean leaves were infiltrated with amitrole. Intact thistle and bindweed plants are capable of producing two metabolites of amitrole. To determine if excised bindweed and thistle leaves were also capable of producing unknown I and unknown II, leaves were placed in petri plates (approximately 10 per plate) and 25 ml. of 0.5 % amitrole was added to each plate. The plates were placed in a vacuum desiccator and a partial vacuum (115 mm. of Hg) was placed on the system for 1 min. This was repeated three times. Following an incubation period of 96 hr, the excised leaves were washed thoroughly and then harvested using the procedure for intact plants. The results were the same for both species. When the incubation time was 24 hr, only amitrole was detected. Both unknowns I and II could be detected chromatographically in both species.

Several methods were attempted to produce unknowns I and II in leaf homogenates. Experiments with ice-water, a phosphate buffer (1/30 M, pH 6.8) and the procedure used to obtain homogenates for catalase activity followed by the addition of amitrole did not result in the production of either unknown I or II. Racuson (11) stated that production of amitrole metabolites in excised bean leaves was reduced in damaged tissue. This suggests that a highly organized, sensitive system is involved in the metabolism of amitrole. This also indicates the products of infiltration of intact leaves were not artifacts caused by the procedure itself.

Rogers (12) postulated one of the unknowns of amitrole metabolism was a glucose adduct. To determine glucose effect on the production of metabolites, amitrole (0.5 %) and glucose (0.25 M) were infiltrated into bindweed leaves. Upon chromatography, unknown I was the only metabolite produced. Identical treatments lacking glucose resulted in the production of both unknown I and unknown II.

The effect of temperature on the transport of photosynthate (3) and P^{32} (7) has been studied using small, temperature-controlled jackets around the organ (petiole or stem) through which transport occurs. Individual petiole jackets which could be maintained at about 4°C were placed in a position to control the temperature of a limited portion of the conducting system, thus permitting study of transport through these jacketed sections to various portions of the intact plant. This type of experiment was done with thistle. Copper tubing was attached to metal jackets having an inside diameter of ca. 3 cm, and when placed over the base of the leaf, covering approximately 4 cm. of the leaf. The ends of the jacket were sealed with non-toxic clay and a solution of ethylene glycol-water was pumped through the jacket to cool the inside air to $4^{\circ} \pm 0.5^{\circ}\text{C}$. Unjacketed controls were maintained at 24°C .

Application of amitrole (50 μg) to the portion of the leaf distal to the jackets resulted in heavy necrosis of the type previously reported (6) of that portion of the leaf in 4 days. This injury was much more extensive than that obtained with the same amount of amitrole applied to a leaf at 24°C and was similar to that obtained with three 250- μg . applications to a single leaf. This experiment was repeated twice using three untreated, jacketed plants and three untreated, unjacketed plants in each experiment for analysis of free reducing sugars of leaves comparable to those of treated plants. The analysis and application of amitrole were made 24 hr. following the placement of the jackets. The content of free reducing sugars increased in leaves with cold jackets at the base of the leaves (Table 3). Although the increase was not greater than the standard deviation because of the rather high variability, the increase appears to be real.

The effect of temperature on the action of amitrole was studied in another manner. As the roots of thistle serve primarily as a storage organ, various temperatures of this root system would be expected to influence transport of carbohydrates to this organ thus altering the normal content of reducing sugars in the leaves. To test this, six pots containing thistle plants were placed in temperature tanks at 15, 25, and 35°C so that only the roots would be maintained at these temperatures. The aerial portions of the plants were

Table 3. *The free reducing sugar content of the distal portions of jacketed ($4 \pm 0.5^{\circ}\text{C}$) and unjacketed ($23.9 \pm 1.0^{\circ}\text{C}$) thistle leaves.*

Temperature	Free reducing sugars (mg/g fresh wt)	
	Series 1	Series 2
23.9C	3.06 ± 1.52	2.64 ± 1.14
4.0C	5.69 ± 1.24	4.81 ± 1.86

Table 4. *The free reducing sugar content of thistle leaves from plants maintained at various root temperatures.*

Temperature	Free reducing sugars (mg/g. fresh wt)
15 C	4.17 \pm 0.24
25 C	2.80 \pm 0.23
35 C	2.20 \pm 0.49

subjected to normal greenhouse temperatures (ca. 24–35°C). After 3 days under these conditions, leaves were removed from three thistle plants, weighed, and the contents of free reducing sugars determined (Table 4). Simultaneously, amitrole (50 μ g) was applied to the same leaf of the remaining three plants. The three plants having a root temperature of 15°C developed discoloration 3 days sooner and to a greater extent than those plants having a root temperature of 25 or 35°C. There was no difference in discoloration between plants at 25 and 35°C.

NaF inhibits the production of unknowns I and II in excised leaves. When NaF (10^{-1} M) was infiltrated with amitrole, the production of both unknowns I and II was completely inhibited, indicating that this metabolic inhibitor had an influence on the metabolism of amitrole. This metabolic inhibitor is also known to inhibit translocation of foliarly applied compounds (2, 7). To determine if NaF could inhibit the transport of amitrole, an application of amitrole (500 μ g) was made with an application of NaF (162 μ g) to the same leaf of bindweed plants. Both compounds were applied alone. Two days following application, the only response was the necrosis of bindweed leaves treated with amitrole and NaF together. There was no response to NaF alone 14 days following application. Amitrole alone resulted in the typical discoloration of the new growth but no necrosis of the treated leaf at the end of 14 days as previously reported (6). The combination of the two materials resulted in necrosis and death of the treated leaf, while there was no discoloration of the new growth.

An additional experiment concerning the movement of amitrole from the treated leaf was performed to ascertain the time factor in translocation. The movement of an applied material out of a leaf can be studied by removal of the treated leaf at various time intervals subsequent to the application of the chemical. This was done with six thistle plants which had been in the light for 12 hr prior to the application of amitrole (10 μ g). Twelve hours after application of amitrole, the treated leaves of three plants were removed. Twenty days following treatment, the intact plants showed discoloration whereas no discoloration was observed in plants having the treated leaf removed 12 hr following application.

Unknown II was produced in substantially larger amounts than unknown I thus facilitating more extensive study of the former. To learn more about the nature of unknown II, bindweed leaves (ca. 30 g.) were infiltrated with amitrole and analyzed by the procedure described previously. The basic eluate was concentrated and chromatographed two-dimensionally with phenol-water (4 : 1) and collidine-lutidine-water (1 : 1 : 2). Spraying the chromatograms with the H-acid reagent indicated the presence of three amitrole-positive spots. These were identified as amitrole and unknowns I and II. Spraying a similar chromatograph with ninhydrin indicated the presence of aspartic and glutamic acids and asparagine in addition to a fourth blue-green spot having R_f values the same as those of unknown II (phenol-water, 0.50; collidine-lutidine-water, 0.23). To remove the amino acids, the basic eluate was stripped on sheets of Whatman No. 1 and chromatographed with phenol-water. The area containing unknown II (R_f ca. 0.5) was cut out, eluted and concentrated. Based on the quantitative procedure for amitrole (6), 133 μg . (amitrole equivalents) of unknown II were obtained. To test the effect of unknown II on thistle, 53.2 μg (amitrole equivalents) were applied to thistle. After 14 days, no discoloration appeared. To test the acid stability of unknown II, a portion of it was refluxed with HCl (6 *N*) for 4 hr. Following neutralization, the acid-treated unknown II was chromatographed with a sample of unknown II prior to treatment. Only one spot identical with unknown II before acid treatment was obtained, indicating the acid did not alter the nature of unknown II. Similar treatment of a mixture of unknowns I and II indicated the disappearance of unknown I. The information with unknown II is in agreement with the recent work of Massini (9) who isolated an alanine derivative of amitrole from plants.

Discussion

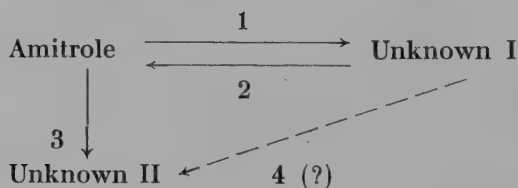
From the results presented, it is possible to establish a working hypothesis concerning the reactions of amitrole in plants. Amitrole in solution penetrates the leaf, at a constant and rather rapid rate (6). After the droplet containing amitrole has dried, penetration into thistle proceeds at a higher rate than that observed with bindweed.

Once inside the plant, the rate of amitrole disappearance in bindweed greatly exceeded that observed in thistle. This was determined both by chromatographic analysis and catalase activity. Therefore, differences in sensitivity between these plants can be explained partially on the low rate of penetration of amitrole once the droplet had dried as compared with thistle. The greater rate of metabolism in bindweed increases the degree of resistance of this plant to the action of amitrole.

Intact bindweed and thistle plants produce two different metabolites from amitrole detectable with H-acid, unknowns I and II. Chromatographically, the unknowns obtained from each species appear to be identical. No attempts were made to determine if these two metabolites were identical with those reported previously (4, 9, 11, 12). Based on the ninhydrin reaction, it is considered likely that unknown II is similar, if not identical, to Carter and Naylor's Compound "1" (4), Massini's ATX (9) and Racuson's "X" (11). This possibility is supported further by the lack of physiological activity against thistle which agrees with the results obtained by Carter and Naylor (5) with their compound "1" on beans.

The metabolic system responsible for the production of both unknown I and II is extremely sensitive to mechanical disruption. Such enzymes, while not unusual, are generally exceptions to the rule. The enzyme(s) responsible for the metabolism of amitrole are inhibited by NaF.

The exact sequence of events cannot be postulated from present information, however, it is reasonable to speculate that the reactions in the following scheme might occur. Reaction 1 leads to the formation of unknown I which can be broken down readily through Reaction 2. Reaction 3 on the other hand is irreversible and leads to the formation of an extremely stable detoxification product. The addition of exogenous glucose favors Reaction 1 and consequently decreases Reaction 3 pushing the equilibrium toward unknown I. It is doubtful that Reaction 4 occurs at all as the



addition of exogenous glucose should thus result in an increase of unknown II rather than the observed inhibition of the metabolite. Environmental conditions favoring a high content of endogenous free reducing sugar, *i.e.*, the petiole of the amitrole-treated leaf cooled to 4°C or low root temperature, in turn result in an increased response to amitrole. This may result from a favorable condition (unfavorable for Reaction 3), for the biosynthesis of unknown I which is either active itself or readily metabolized (Reaction 2) to amitrole. The relative instability of unknown I supports the possibility of its metabolism to amitrole. It is quite likely that the greater sensitivity of thistle to amitrole as compared to bindweed is a consequence of the more favorable condition in bindweed for the occurrence of Reaction 3.

Up to 80 % of the amount of amitrole applied penetrates the leaf in 12 hr

(6). However, no detectable movement of amitrole has occurred within 24 hr as shown by excising the treated leaf. To account for the observed time lag between penetration and movement from the treated leaf, we postulate that amitrole must undergo the chemical reaction forming unknown I before it can be translocated from the treated leaf. This is supported by the observed time (more than 24 hr.) required to synthesize unknown I by the excised leaf, also, by the inhibitory effect of NaF on translocation of amitrole and the synthesis of unknown I.

This hypothesis, describing a chemical combination of the active molecule with a carrier, quite likely a carbohydrate, provides an attractive hypothesis for the chemical basis of systemic action, and it may well be, that for a molecule to be transported in the phloem from the treated leaf to some distal portion of the plant, it must undergo such a chemical combination or reaction.

Summary

Canada thistle and field bindweed produce two metabolic derivatives from amitrole. These compounds, referred to as unknowns I and II, appear to be the same for both species. Conditions which favor an increase in the free-reducing sugar content of the leaves to which amitrole is applied appear to favor the production of unknown I. Under these conditions the formation of unknown II may not be favored. Unknown II does not appear to have phytocidal activity. A scheme for the biosynthesis of unknowns I and II and the equilibria between these compounds and amitrole is postulated. It is also postulated that amitrole must undergo a chemical reaction, possibly to unknown I, in order for translocation to occur.

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Studies on Plant Antitranspirants

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Introduction

The main loss of water from a field covered by a crop and receiving rational irrigation is due to plant transpiration. Loss of water by soil evaporation becomes negligible after the top layer has dried out; this is particularly so in arid and semi-arid climates (14). It follows that any attempt to reduce the water loss from an irrigated field should be directed at the reduction of transpiration. This raises the long discussed question as to whether or not transpiration fills any vital function in plant development. Recent work indicates that it is of little if any importance (2, 17) although results of at least one investigation show that under conditions of 100 % humidity, growth may be reduced (21). Opinion on the deleterious effects of the greater part of transpiration, in dry climates, is however undivided.

There are a number of possible practical approaches to the problem of the reduction of transpiration, such as the direct effect of certain ions (16), the effect of hydrophobic monomolecular films obtained from higher alcohols or silicones (5) and the effect of sprays forming water-vapour impervious films, such as was emulsions (15) and emulsions of plastic compounds (6). One of the main problems to be tackled has been the development of a suitable anti-transpirant which is selectively permeable (15).

To date most efforts to reduce transpiration have been made by horticulturalists wishing to prevent the dessication of trees and shrubs during transplantation (6, 1, 7, 13). A very few papers deal with the effect of antitranspirants on field crops (3), and indicate unfavourable effects on photosyn-

thesis. However, results of critical evaluations have been very inconsistent and inconclusive.

As the number of plastic materials potentially of use as antitranspirants has increased vastly in recent years, it seemed worthwhile to reconsider this subject. In preparation for this work a considerable number of compounds were examined with a view to selecting one which would approach the following concept of an ideal antitranspirant: a substance which could be easily applied as a non phytotoxic spray, would form a continuous film on the leaf surface, impermeable to water vapour but allowing diffusion of CO_2 and O_2 , would be resistant to weathering — particularly to the destructive effects of intense insolation and micro-organisms — and, finally would be inexpensive. In the investigation reported here we aimed at the elucidation of the effects of those compounds whose characteristics approach the above, on transpiration and on the plant development in general.

Materials and Methods

Phaseolus vulgaris var. Bulgaria plants, grown to the two cataphyll leaf stage were used for most of these experiments, as they are easily grown and are very sensitive to phytotoxic substances. Furthermore, there is little difficulty in obtaining a good spray coverage on their glabrous cataphyll leaves, and they transpire very rapidly in hot dry weather. Other plants were also used occasionally, but results quoted refer to *Phaseolus*, unless otherwise stated.

An indication of the photosynthetic activity of the cataphyll leaves was obtained by following the dry matter formation of the newly developing trifoliolate leaves. This was permissible only while the latter leaves were still small — before the development of their own photosynthetic surface. In field experiments the usual method of following relative growth rates was adopted.

Transpiration was measured by repeated weighings of potted plants. The pots were enclosed within polyethylene bags, which were firmly fastened around the stems of the plants.

Stomatal apertures were measured by Lloyd's technique (11) which was found to be quite satisfactory for the plant material examined.

Phytotoxic effects were evaluated by visual observation.

Apparent photosynthesis under varying micro-climatical conditions was measured by an adaptation of the methods of Heinicke (10) and Verduin and Loomis (20). One important modification was that the flow meters were placed between the plant containers and the absorption columns and not between the columns and the air pumps (20). In the latter position the air pressure is less than atmospheric and varies with the height of the foaming fluid in the absorption columns and with the resistance of the apparatus — mainly due to the sintered glass filters, thus causing highly erroneous readings in the flow meters, which are calibrated at constant atmospheric air pressure. Three units were used. Two of the units were connected to glass containers, 36 cm. high and 30 cm. in diameter, the third led to the free air. Five potted plants could be placed in each of the two containers. The pots were

covered with polyethylene, which was shown experimentally, to prevent soil gas exchange from interfering with the experiments. The containers were placed in such a position as to be in the sun in the morning hours and in the shade from noon onwards. The temperature within the containers could be reduced by about 8°C by means of water sprayed on the outside of the glass. Conditions of low humidity could be obtained within the container by means of removable racks of calcium-chloride. Air was passed over the plants at a rate of 47 litres per hour. Relative humidity within the containers was measured by a calibrated hair hygrometer, temperature by a shaded mercury thermometer.

The three units were first run for one hour without plants to ensure their equilibration; next, 10 plants were grouped in pairs by size and the two equal groups placed in two of the units. Photosynthesis of these two groups was then determined under optimal conditions of humidity, light and temperature. The third unit was run as a blank throughout. The ratio of the photosynthesis of the two groups was noted. It rarely exceeded 1.1. One of the groups was sprayed and the photosynthesis of the two groups was then measured under varying conditions of insolation, temperature and relative humidity; the results being corrected by the above ratio.

By testing relatively large groups of treated and control plants, simultaneously, under known conditions, variability was very much reduced.

The antitranspirants were applied to the plants as sprays. Low volume, high pressure spraying was found to be the most effective for the formation of an even, continuous film on the leaf surface. Spraying during dry hot weather was found to be superior to spraying in a cool, humid, atmosphere, as in the former case the spray material does not have time to penetrate the leaf but rapidly dries on the surface, thus reducing the risk of phototoxicity.

A large number of compounds and formulations were screened for possible use as antitranspirants. The results of these experiments will be published elsewhere. Unless otherwise stated the compound S-789 a copolymer dispersion of vinyl acetate-acrylate esters, produced by Serafon Co. Rehovot, was used in these experiments.

Results

Measurements of the effect of different compounds on transpiration and growth during the hot summer season gave results which, at first, were difficult to understand. Compounds which in laboratory tests considerably hindered the diffusion of water vapour and CO₂ not only did not reduce transpiration, but sometimes even slightly increased the water loss when measured over a period of a few days. This was particularly difficult to understand as during the hot hours of the day the treated plants appeared to be fully turgid, or loss of turgor was greatly delayed, while the control plants became flacid a short while after being transferred from the greenhouse to the open air — even when the containers were fully watered. Moreover, despite the impeded passage of CO₂ the treated plants very often showed more vigorous growth than the controls (Table 1).

Table 1. *The photosynthetic activity of cataphyll leaves of Phaseolus after treatment with an antitranspirant.* Photosynthetic activity is expressed by the dry matter formation of the trifoliolate leaves during 7 days (see methods). Before spraying with S-789 at 9 % T. S. (Total Solids), plants were paired according to size. The weather during this experiment was particularly hot and dry — temperatures in the region of 30°C. R.H. not above 40 %.

Pair No.	Dry Matter mg.	
	Control	Treated
1	218	128
2	88	197
3	71	149
4	49	242
5	69	326
6	32	256
\bar{x}	88	216

Measurements of transpiration made over short periods of time, using plants transferred from the cool greenhouse to the hot sun and sprayed with substances having an effect similar to the above on transpiration and growth showed extreme fluctuations (Table 2).

As can be seen from the results given in Table 2 there was a difference between the march of transpiration of the control and the sprayed plants.

Table 2. *Effect of an antitranspirant spray on transpiration of potted lettuce plants.* Time given is from moment plants were transferred from the shade to hot sun. Before spraying, plants were paired according to transpiration rates as measured on the previous day.

Treatment	Pair No.	Transpiration in grams per plant in period			Total Transpiration during the 210 minutes:g
		0-30-min.	30-90-min.	90-210-min.	
Control	1	2	6	11	
	2	4	7	20	
	3	11	3	13	
	4	3	3	10	
	5	4	8	5	
\bar{x}		4.8	5.4	11.8	22.0
Calculated transpiration - g/hr.		9.6	5.4	5.9	
Goodrich Latex -652- 20 % T.S. (Total Solids)	1	1	4	6	
	2	0	8	10	
	3	6	15	20	
	4	0	16	12	
	5	1	13	9	
\bar{x}		1.6	11.2	11.4	24.2
Calculated transpiration - g/hr.		3.2	11.2	5.7	

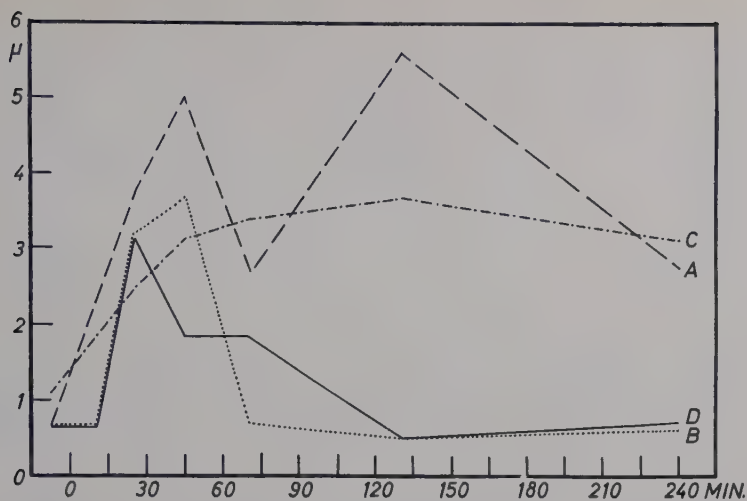


Figure 1. *Effect of an antitranspirant spray on stomatal aperture of potted lettuce plants.* Pots were well watered and the plants sprayed with Goodrich Latex 652 20 % T.S. A: lower epidermis treated, B: control, C upper epidermis treated, D: control. On the abscissa time after transfer from shade to sunlight, on the ordinate stomatal aperture.

We suspected that this difference was due to the varying behaviour of the stomata and therefore in a later experiment under very similar conditions, the stomatal apertures were measured at short intervals. The results are given in Figure 1.

Comparison of Table 2 and Figure 1 suggests the following explanation for the relative transpiration rates of the control and sprayed plants. At first the stomata of both the controls and the sprayed plants were open and in this period the antitranspirant reduced the transpiration rate of the treated plants. In the second period the stomata of the control plants closed hydroactively, as the transpiration was greater than the water supply from the roots, while the stomata of the treated plants remained wide open. This reduced the transpiration of the control plants to below that of the treated plants. In later hours the stomata of the treated plants also closed somewhat, thereby reducing the transpiration of the treated plants to the level of the controls.

The early hydroactive closure of the stomata and its delay by an antitranspirant spray, was not limited to potted plants, nor to one species of plant, nor to one type of antitranspirant. For example, compound S-789 had such an effect on the stomatal aperture of bean plants growing in a well irrigated field (Figure 2).

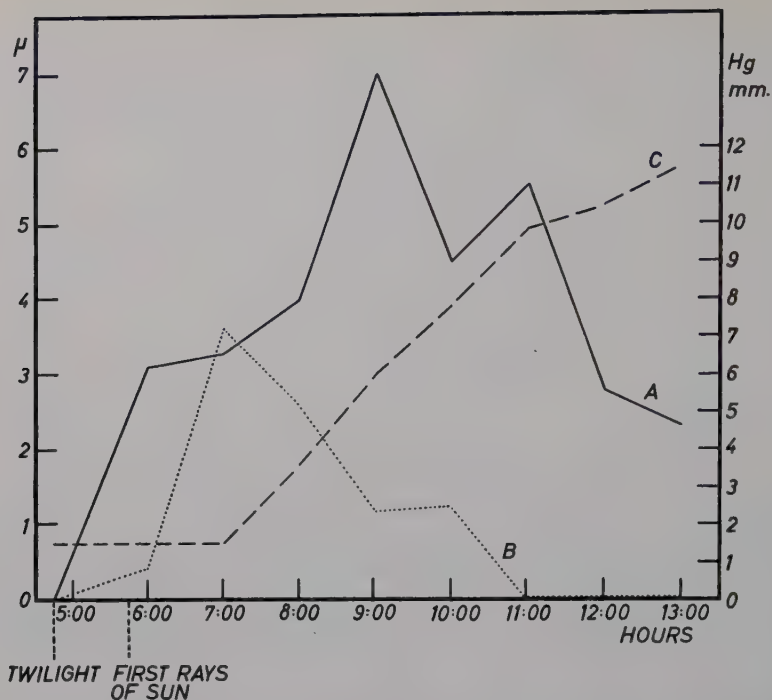


Figure 2. *Effect of antitranspirant on stomatal aperture of bean plants growing in the field.* Plants sprayed with S-789 6.25 % T.S. Field well irrigated. Stomatal apertures of lower epidermis only are given (left ordinate). Vapour pressure deficit (right ordinate) was calculated from ambient air temperature and relative humidity only. Actual leaf temperature and relative humidity within mesophyll space were neglected. On the abscissa hours of day. A: treated, B: control, C: vapour pressure deficit.

In order to find an explanation for the increased growth of the treated plants (Table 1) and in order to learn the effect of the delayed stomatal closure on photosynthesis, the relative rates of apparent photosynthesis of control and treated plants were measured under varying micro-climatical conditions. Representative results of a large number of such measurements are given in Table 3.

We did not succeed in simulating conditions of low relative humidity at moderate temperatures. However, while at high relative humidities apparent photosynthesis was reduced by the treatment, under adverse conditions the reverse effect was observed. A clear and significant increase in photosynthesis at high temperatures and low relative humidities resulted from treatment of plants with the antitranspirant.

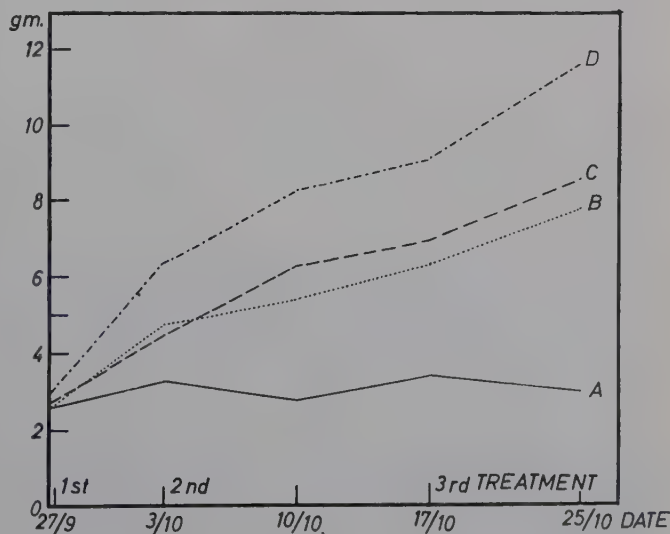
The varying effects described above made it interesting to study the

Table 3. *Effect of an antitranspirant on apparent photosynthesis under varying micro-climatical conditions. Plants sprayed with S-789 9 % T.S.*

Conditions in plant containers	Stomatal Aperture μ				Effect of antitranspirant on apparent photosyn- thesis. Relative to control plants
	Control Plants		Treated Plants		
	Before Test	After Test	Before Test	After Test	
Shade, Relative Humidity 90 % Temp. 25 — 29°C	3.0	3.7	4.4	3.7	Reduction by 70 %
Sun, Relative Humidity 90 % Temp. 34°C	2.9	4.4	3.7	5.0	Reduction by 25 %
Sun, Relative Humidity 35 % Temp. 37°C	1.5	3.1	3.7	4.4	Increase by 14 %

overall effect of an antitranspirant on plant growth under field conditions over a more protracted period. Four blocks, $6 \times 1\frac{1}{2}$ m. were sown with beans. Two blocks were given frequent and adequate irrigation and 2 received only half this amount. No rain fell during the period of this experiment. Each block was subdivided into 4 plots, two of which were sprayed with the S-789 antitranspirant, the remaining two being controls. At weekly intervals samples of 6 plants each, were taken at random from each of the 16 plots, and the dry weights were noted. Average weekly data from the 4 replications of each of the 4 treatments are given in Figure 3.

Figure 3. *Effect of antitranspirant spray on growth of bean plants in the field.* The plants were sprayed thrice with S-789 at 6.25 % T.S. The co-ordinate for 25/10, for the treated, half irrigated plots has been estimated after loss of one of the 4 plots. A: half irrigated control, B: treatment, C: fully irrigated control, D: treatment. On the abscissa date of sampling, on the ordinate dry weight g. as average of four replicate plots.



Results of the field trial show that there was only a slight difference in the dry matter production of the half irrigated treated plots and the fully irrigated control plots. In fact, therefore, the water requirement per unit dry matter produced was smaller in the treated plots than in the controls. Moreover, even in the fully irrigated plots the antitranspirant caused a rise in dry matter production.

Discussion

As previously mentioned, early investigations of the effect of antitranspirants gave very inconsistent results. The data of the experiments presented above suggest an explanation of these divergencies, on the basis of the interaction of the plant with soil-moisture, antitranspirant and atmospheric conditions.

The only reported field trial of the effect of an antitranspirant on photosynthesis was conducted during a season in which atmospheric conditions were never such that the roots were unable to supply the leaves with the water transpired (3). Under these conditions, as could be expected from the results of the experiments reported above (Table 3), photosynthesis was seriously reduced.

If the very favourable effect of the antitranspirant on growth under field conditions (Figure 3) is compared to the figures obtained for apparent photosynthesis (Table 3) it seems that the increased apparent photosynthesis under hot dry conditions was much less than was to be expected. This may be because the net photosynthesis was partly masked by a very much increased respiration, caused by high temperatures $\sim 35^{\circ}\text{C}$ in the containers. Aeration may also have been inadequate, resulting in reduced convective cooling of the leaves. In addition the carbon-dioxide content of the air in the containers may have fallen during the experiment.

The increase of transpiration by an antitranspirant spray reaches a maximum when the stomata of the sprayed plants are fully open and those of the control closed. If the antitranspirant is further improved, by reducing its permeability to water vapour, transpiration of sprayed leaves can only decrease, so that it becomes less than that of the controls — under all atmospheric conditions. This in effect is what has been obtained with the compound S-789 which in no case increased transpiration, but nearly always decreased it. The same is true in respect of CO_2 . An ideal antitranspirant — one which allows free diffusion of CO_2 — would not decrease photosynthesis even under cool, humid, conditions.

It should be emphasised that in spite of the very encouraging results obtained, compound S-789 is still far from ideal, being only partly impervious to water vapour and constituting a considerable barrier to CO_2 diffusion.

The phenomenon of early closure of stomata even under conditions of adequate soil moisture is quite common in hot climates (12, 18). Although other reasons have been suggested (9), this early closure is probably mainly due to atmospheric drought and results in reduced transpiration which can be correlated to reduced photosynthesis (4).

It is uncertain whether the increased photosynthesis when stomata are open, — as observed here in the treated plants — is due solely to easier diffusion of CO_2 , or also, or mainly, to the concomittant improved protoplasmic hydrature (8,19). The overall effect is however very significant and it seems likely that in arid climates improved antitranspirants should not only reduce water expenditure and increase drought resistance, but may also enhance yields of irrigated crops.

Summary

(1) The effect of antitranspirants on transpiration, photosynthesis and growth under varying field and controlled laboratory conditions was assayed.

(2) Under hot dry conditions an antitranspirant which is not very impermeable to water vapour will not only not reduce transpiration, but may even increase it by delaying or preventing early stomatal closure. An improved antitranspirant had this effect on the stomata without increasing water loss.

(3) Under simulated mild atmospheric conditions, antitranspirants at present available were shown to reduce photosynthesis while under simulated hot dry conditions, photosynthesis and growth were increased.

(4) Results of a small scale field trial, carried out under hot dry weather conditions, showed reduced water expenditure per unit dry matter produced and increased dry matter production under conditions of both adequate and inadequate soil moisture.

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Kinetics of the Auxin Catabolism

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Introduction

Several mechanisms have been suggested to interpret enzymatic oxidation of indole-3-acetic acid (IAA) by plant extracts or purified enzymes (Ray 1958 and 1961, Pilet 1961 a, p. 319–344, Pilet 1960 d). The proposed theories differ only in a few details. Up to now, not many works (see MacLachlan and Waygood 1956 a) have been devoted to kinetics of the IAA oxidation, and yet analysis of the rates of IAA destruction can bring out useful indications of the distinct mechanisms which contribute to the IAA oxidation. This paper is a preliminary account of the physiological analysis of the kinetics of IAA metabolism.

Biological Material

The seeds of *Lens culinaris* Med. were first soaked in deionized water for 12 hours, then washed, and finally placed on wet filter paper in Petri dishes in darkness at $22 \pm 0.5^\circ\text{C}$. The first selection was made after 24 hours and only seedlings measuring 3 ± 1 mm. were kept and replaced in the above conditions. The seedlings were removed for treatment when they had reached a length of 18 ± 2 mm., for this has been found to be a period of optimal growth (Pilet 1951 a and b, Pilet and Went. 1956). Most experiments reported below were made with root tip preparations measuring 3 mm; fragments were used as the source of active enzyme.

Methods

The essential part of the technic has been presented in a previous paper (Pilet 1957 b); it has been taken up again recently and improved (Pilet and Collet, in press). A summary of the methods used for our assays follows.

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Enzymatic extracts

In vitro IAA destruction experiments were performed by first converting the tissues (6 mm tips) of the 18 mm. Lens root to a "brei". The tissues were removed from the medium by decanting, rinsed in a few ml. of the buffered solution ($\text{Na}_2\text{HPO}_4 - \text{KH}_2\text{PO}_4$; pH 6.1) and transferred to a previously chilled mortar containing SiO_2 and cold buffer. They were then ground and the mixture subjected to a first centrifugation (3.500 g; 15 min.). The solid extract is added to the buffered solution for a second centrifugation (8.000 g; 10 min.). Then the supernatants were decanted into 10 ml. volumetric flasks and made up to volume with buffer.

Enzymatic incubation

Ten ml. of the solution (enzymatic extract) were then mixed with 10 ml. deionized water and 20 ml. of cold buffer. At zero time, 10 ml. of IAA (aqueous solution containing 50 $\mu\text{g}/\text{ml}$. of IAA) were added. The active mixtures were incubated in a metabolic shaking incubator, thermostated at 28°C in complete darkness. All the operations were made in green light.

IAA destruction

Initial and residual IAA concentrations at various times of the enzymatic incubation were determined by colorimetric analyses. Colorimetric determination of IAA was based on the observation of Salkowski: small amounts of IAA form an intense and typical red colour with FeCl_3 in the presence of concentrated H_2SO_4 (see Tang and Bonner 1947). This reaction may be determined by quantitative colorimetry. The technic used in the present investigation is as follows: 2 ml. of the active mixture, containing (at time 0) 10 μg of IAA per ml., are pipetted into a test tube containing 8 ml. of our reagent (Pilet 1957 b).

- 3 ml. of 1.5 M $\text{FeCl}_3 - 6 \text{ H}_2\text{O}$ (pro anal; MW 270.32)
- 60 ml. of H_2SO_4 (1.84; MW: 98.08; 95 - 97 %))
- 100 ml. of distilled and deionized water.

Expression of the results

The IAA catabolism is expressed in terms of IAA destroyed after a certain time of enzymatic incubation (from 0 to 70 min.) and for 0.1 mg of protein nitrogen (Pilet and Siegenthaler 1959).

Preliminary Analyses

First experiments were performed with different kinds of preparations from Lens roots. A series of cytological analyses has shown that the tip composed essentially of older cells is located between the extreme point and 0.5 mm. from it, while the region containing the young cells (essentially the meristem) is situated in the region from 0.5 to 3.0 mm. In order to prepare

Table 1. *Auxin content (acid fraction free auxins) and intensity of auxin catabolism of root segments. Lens root tip, of 18 mm. used. Old cells: root extreme tip from 0.0 to 0.5 mm., young cells: root meristematic region from 0.5 to 3.0 mm.*

Expressed as :	Auxin content in μg of IAA		Auxin catabolism in μg IAA destroyed/60 min	
	old cells	young cells	old cells	young cells
Per 100 mg. of fresh weight	0.13	21.00	75.0	21.0
Per mg. total N	0.12	24.10	73.0	24.0
Per mg. protein N	0.16	70.61	90.0	71.0
Per 10^8 cells.....	1.26	86.38	73.3	8.6

these two root sections, a small guillotine was developed (Pilet 1959 a). Except for the first experiment, all analyses reported below were made with extracts from 3 mm. root fragments (root tip). Table 1 summarizes the results obtained from the two types of extracts. The data show that IAA destruction is greater in old than in young tissues.

These observations confirm our preceding results and also those previously reported by Galston and Dalberg (1954): "the ability of pea seedlings' cells to destroy the native auxins increases as the cell ages." The suggestion was made (Pilet 1957 a) that IAA-oxidase determines the endogenous auxin level: high enzyme activity meaning low auxin content (old cells) and vice versa. However, recent observations (Pilet 1961 b) do not exactly confirm this hypothesis. If the IAA destruction is expressed in terms of growth gradients (Pilet 1960 c and 1961 c, Pilet and Siegenthaler 1961), simple relations between auxin catabolism and auxin content are not so evident. In another way, however, the fact that IAA destruction increases with age would seem to contradict previous results, which show that the endogenous auxin concentration of complete roots increases with increasing age (Pilet 1951 a and 1953).

Nevertheless, it can be supposed that two processes operate at the same time: the root tissues greatly increase their ability to destroy native auxins in proportion to the age of the roots simultaneously as the accumulation of auxins is increasing. Even if the destruction increases, the final auxin content rises because auxins are produced faster than they are destroyed. These hypotheses suggest a mechanism of enzyme induction which was previously observed (Galston and Dalberg 1954, Pilet 1959 b). Consequently, the following scheme can be suggested (see Figure 1):

- 1) initial IAA level (IL)
- 2) enzymatically-catalysed oxidation of IAA occurs: initial IAA-enzymes activity (IE), initial IAA-concentration destroyed (ID) and non-destroyed IAA concentration (ND)

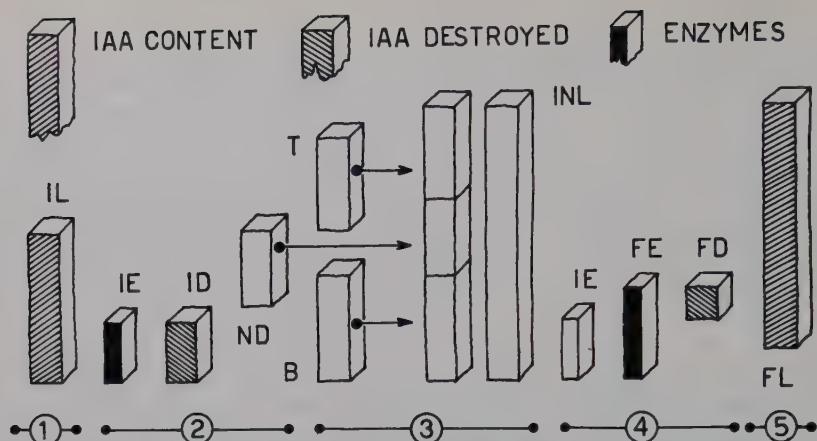


Figure 1. Relations between auxin content and auxin catabolism in the roots (see p. 789).

- 3) simultaneously IAA is translocated from other part of the organ (T) and IAA is *in situ* biosynthesised (B); consequently the endogenous IAA concentration increases on an IAA intermediary level (INL)
- 4) IAA-enzyme induction takes place and the activity of these enzymes increases till a final IAA enzyme activity (FE) which produces an IAA destruction (FD)
- 5) consequently IAA concentration decreases (IAA oxidation rate increases) till a final IAA level (FL)

Phases of Enzymatic IAA Destruction

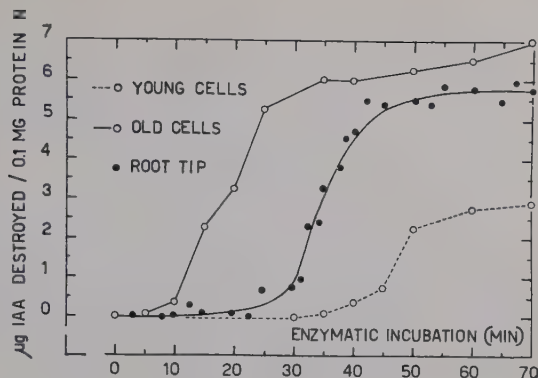
Study of the progress of IAA destruction for the three types of enzymatic extracts shows (see Figure 2) that in the case of preparation from old cells (root cap: 0.0–0.5 mm.) the decomposition of IAA is almost immediate, although for the young cells (meristem: 0.5–3.0 mm.) there is a certain lag time. If we consider the IAA destruction by root tip extracts, a similar curve is obtained.

From the preceding observations it can be concluded that IAA destruction occurs following three distinct phases:

- 1) a *lag period*, during this time the rate of the IAA destruction is small;
- 2) an *activation phase*, during this period the rate increases up to a maximum;
- 3) an *equilibrium state*, during this step the rate becomes constant.

Previous experiments with IAA labeled with C^{14} and root-tip extracts gave the same results (Pilet 1960 a and b). Curve of IAA destruction expressed in terms of per cent detected (the paper was drawn through a counting chamber at constant rate, under slight positive pressure; counting pulses were amplified

Figure 2. Phases of enzymatic IAA destruction in relation to time. Active extracts prepared from root tip (0-3 mm.) and from root cap (old cells: 0.0-0.5 mm.) and meristematic region (young cells: 0.5-3.0 mm.).



and directly recorded by a rate meter) on the chromatogram for R_f values, similar to those of IAA, indicates that:

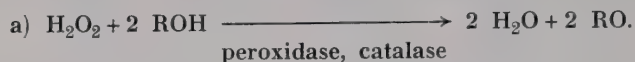
- 1) between 0 to 30 min., IAA undergoes weak decomposition but destruction speed increases with increasing time of incubation,
- 2) between 30 to 45 min., IAA destruction is maximal,
- 3) between 45 to 90 min., IAA undergoes further destruction but more and more slowly,
- 4) from 90 min. on, decomposition of IAA is still to be noted but inactivation speed becomes practically steady.

Using IAA-oxidising enzyme of *Omphalia flavidus*, Ray (1961) observed the following kinetic phases of enzymatic IAA oxidation: a) an induction phase (from 0 to 10 min.), b) a steady state (from 10 to 20 min.), c) a period which can be characterised by secondary effects (from 20 min.).

As it can be seen, except for the duration of these phases, the kinetics of IAA destruction are practically identical for *Lens* and for *Omphalia* extracts.

According to Ray (1961), the lag or induction phase which precedes attainment of a rapid reaction rate appears to be due to the fact that the reaction medium does not initially contain concentrations of oxidation intermediates great enough to allow a substantial rate of destruction. To-day the interaction of H_2O_2 and IAA-oxidising enzyme is well known. Goldacre (1951), then Galston, Bönner and Baker (1953), supposed that H_2O_2 is used in a peroxidase-catalysed step and re-formed from O_2 in oxidase-catalysed phase. MacLachlan and Waygood (1956 a and b) suggested that IAA is oxidised by Mn^{+3} with the formation of a radical which reacts with O_2 . The action of manganese on the enzyme's activity insuring the destruction of IAA has been studied subsequently (Hillmann and Galston 1956, Pilet 1957 a). Recently Waygood and MacLachlan (1961) have discussed their previous theory pro-

posing the following mechanism whereby a redox catalyst (ROH) and catalase or peroxidase could interact to oxidise manganese:



The reaction (a) is similar to that proposed by Andreae (1955) and the reaction sequence (b) and (c) are the reactions first described by Kenten and Mann (1950). The action of H_2O_2 and the induction phase and the rate of the IAA-destruction during the steady state will be discussed a little further.

Expression of the Kinetics of IAA-destruction

With a view of studying the action of various factors on the three phases of IAA destruction and the rate of IAA oxidation, we suggest (see Figure 3) determining the following:

- 1) the *lag period* (LP) which corresponds to a weak destruction of IAA (phase 1)
- 2) the *natural tangent* α which is measured on the curve giving the IAA destruction at the middle of the activation period (phase 2).

Biokinetic Analyses

Results of the experiments concerning the enzymatic IAA destruction are analysed according to the directions given by Tang and Bonner (1948).

If E indicates the enzyme and S the substrate, according to the earlier papers of Michaelis and Menten, and Lineweaver and Burk (see Bladergroen 1955), this formulation may be expanded:

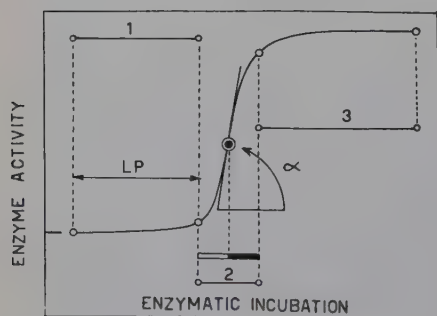
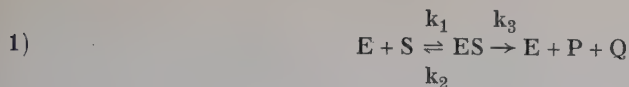


Figure 3. Kinetics of IAA destruction. Phase 1: lag period (LP) — phase 2: activation period — phase 3: equilibrium state.



Then, the rate of the reaction as a function of enzyme and substrate concentrations can be obtained:

$$2) \quad v_1 = k_1 \cdot [E] \cdot [S]; \quad v_2 = k_2 \cdot [ES]; \quad v_3 = k_3 \cdot [ES]$$

$$3) \quad \frac{[E] \cdot [S]}{[ES]} = K_s = \text{constant of Michaelis}$$

Supposing that $[e]$ is the total amount of enzyme, we have:

$$4) \quad [E] = [e] - [ES] \quad \text{and} \quad 5) \quad [ES] = \frac{[e] \cdot [S]}{K_s + [S]}$$

We can also use the real (v) and the maximal (V) velocity:

$$6) \quad v = k_3 \cdot [ES] \quad \text{and} \quad 7) \quad V = k_3 \cdot [e]$$

Combining these equations, we obtain:

$$8) \quad v = \frac{V \cdot [S]}{K_s + [S]}$$

In Figure 4 A, variations of v are plotted as a function of IAA concentration ($[S]$). As it can be seen, with the present system of enzyme, V is $0.7 \mu\text{g} \pm 0.1$ of IAA destroyed per min. and K_s value is $30 \mu\text{g} \pm 6$ of IAA per ml.

The double reciprocal of velocity and substrate concentration is expressed in the following equation:

$$9) \quad \frac{1}{v} = \frac{K_s}{V} \cdot \frac{1}{[S]} + \frac{1}{V}$$

It can be observed (see Figure 4 B) that the reciprocal of the rate of the IAA destruction is a linear function of the reciprocal of IAA concentration.

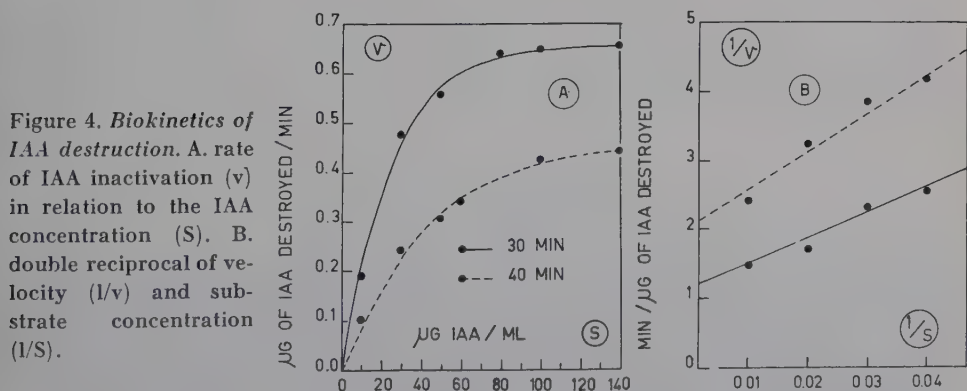


Table 2. *Lag period and tangent α of the activation curves of the IAA destruction in relation to enzyme and substrate concentrations.* 2 ml. of enzymatic extract (different concentrations), 6 ml. of buffer solution (pH: 6.1), 2 ml. of IAA (different concentrations). Temperature: $28^{\circ}\text{C} \pm 1$. Enzyme concentration: mg. fresh weight/10 ml. (2 ml. of IAA at $50 \mu\text{g/ml}$). Substrate concentration: μg of IAA/10 ml. (2 ml. of enzymatic extract at 5 mg. fw/ml.).

Concentration	Lag period in min.	Natural tangent α
Enzyme		
50	39.7 ± 2.9	1.50
100	31.2 ± 1.7	2.06
200	4.8 ± 1.2	3.87
Substrate		
10	34.3 ± 3.0	0.46
50	30.8 ± 1.9	2.32
100	6.5 ± 1.4	2.45

Enzyme and Substrate Concentrations

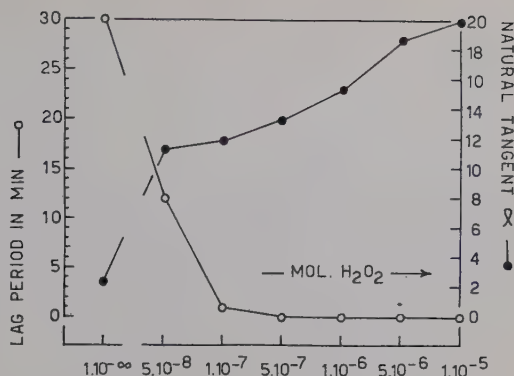
The relation between the duration of the lag period and the rate of inactivation of IAA on the one hand and, on the other, the concentration of the enzyme (expressed in terms on mg. of fresh weight root tip per 10 ml.), is shown in the experiment recorded in Table 2. The results indicate that at low concentrations of enzyme, rate increases with increasing concentration of the enzyme, while at higher concentration practically no change of the rate of IAA destruction occurs; as it will be seen below, the substrate becomes the limiting factor. On the other hand, with increasing level of the enzyme, the lag period decreases also, but not proportionally.

The parallel data (see Table 2) show that lag period decreases with increasing IAA concentration and that the rate of IAA destruction increases with increasing amount of IAA; the substrate concentration is no longer limiting at concentrations above approximately $50 \mu\text{g}/10 \text{ ml.}$ of IAA under the conditions of these experiments.

Oxidation Intermediates

The probable role played by H_2O_2 in the mechanisms of IAA destruction was discussed above. In Figure 5 it can be seen that the IAA destruction by the root tip extracts can be considerably modified by addition of H_2O_2 to the active mixture. If H_2O_2 is added initially, no induction phase is observed, except for a very low H_2O_2 concentration. The rate of IAA destruction,

Figure 5. Action of H_2O_2 on the IAA destruction. Active mixture: 2 ml. (5 mg. fw/ml.) — buffered solution: 4 ml. (pH: 6.1) — H_2O_2 solution: 2 ml. — IAA solution: 2 ml. (50 μ g/ml.).



expressed in terms of natural tangent α , increases with increasing concentration of added H_2O_2 . Ray (1961) presented the same observations; he noted that the rate increases with increasing H_2O_2 concentrations up to a maximum, which he calls the H_2O_2 saturation rate, and which he presumes is due to saturation of the peroxidase with H_2O_2 . In his experiments with *Omphalia* extracts, this concentration is about 2.10^{-6} M H_2O_2 , and we found for *Lens* extracts a concentration approximately equal to 5.10^{-7} M. Consequently the idea of a unique oxidase — responsible for the IAA destruction — is no longer motivated. As it was already discussed, this mechanism appears to be a free-radical one, but (see Ray 1960) “the nature of the interactions is such as to make difficult a decision as to whether H_2O_2 is a reaction intermediate”; but certainly H_2O_2 exerts typical effects upon the reaction rate.

Action of Effectors

The action of the *Lens* root tip enzymes in destroying IAA can be stimulated or inhibited by several substances (Pilet 1957 a and c and 1961 b); in the present paper results will be discussed for two different products: 2,4-dichlorophenol (DCP) and gibberellic acid (GBA).

2,4-Dichlorophenol

It is now generally agreed that various monophenols enhance the enzymatic destruction of IAA (Goldacre 1961, Kenten and Mann 1950, Millmann and Galston 1956, Pilet 1957 a). The data to be presented below concern experiments with DCP which is used at various concentrations (1.10^{-5} to 5.10^{-3} M). Figure 6 summarises the results concerning the action of the DCP

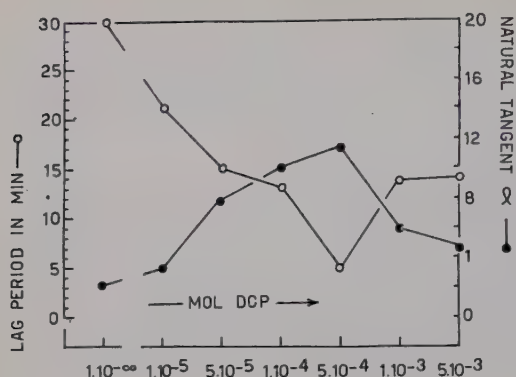


Figure 6. Action of 2,4-dichlorophenol (DCP) on the IAA destruction. Active mixture: 2 ml. (5 mg. fw/ml.) — buffered solution: 4 ml. (pH: 6.1) — DCP solution: 2 ml. — IAA solution: 2 ml. (50 μ g/ml.).

on the IAA-destruction. It can be seen that the rate of IAA inactivation increases with increasing DCP concentration up to a maximal level which occurs, under the present conditions, at 5.10^{-4} M. On the other hand, DCP produces a decrease of the lag period up to the same optimal concentration.

Gibberellic acid

The effect of GBA on plant tissue growth seems to be caused by some process involving auxin, as already suggested by Brian and Hemming (1958), Kuse (1958) and Galston and Warburg (1959). Pilet (1957 c) observed that GBA produces a decrease of the IAA destruction by carrot tissue extract and, consequently, it can be supposed that GBA causes an inhibition of the IAA-destroying enzyme activity. Similar results were obtained by Galston (1957) with pea extracts. Using different sources of enzymes, Pilet and Wurgler (1958), Pilet and Collet (1959) observed again that GBA altered the IAA catabolism with a reduction of the IAA destruction and the probable interaction of 3-hydroxy-methoxy-cinnamic acid. Stutz and Watanabe (1957) confirmed

Table 3. Lag period and tangent α of the activation curves of the IAA destruction in relation with *in vitro* action of gibberellic acid at different concentrations. 2 ml. of enzymatic extract (5 mg fw/ml), 4 ml. of buffer solution (pH: 6.1), 2 ml. of gibberellic acid (different concentrations), 2 ml. of IAA (50 μ g/ml.). Temperature: $28^{\circ}\text{C} \pm 1$.

Gibberellic acid concentration μ g/ml	Lag period in min.	Natural tangent α
0	30.0 ± 3.5	2.15
1	28.5 ± 2.7	2.36
10	25.3 ± 2.8	2.84
50	24.7 ± 2.1	3.71
100	24.6 ± 2.3	6.50
500	23.4 ± 2.2	8.12

these observations and Nitsch (1957 and 1959) reported that GBA treatment of some plants increased their auxin content. However, experiments of Kato (1961), using combinations of GBA and IAA, have suggested that the effects of GBA involve a physiological sequence different from that of auxin (see also Kato 1958, Kato and Katsumi 1959).

Table 3 shows how the lag period and tangent α are modified with GBA treatment. If the concentration increases, the lag period decreases obviously; however the natural tangent extends in the same way. These observations show that GBA accelerates enzymatic destruction of IAA (reduction of the induction phase and increase of the rate of destructive process).

Physical Factors

pH

Analyses of the action of the pH on the enzyme reaction were made with the buffered solution ($\text{Na}_2\text{HPO}_4 - \text{KH}_2\text{PO}_4$). For this experiment, 2 ml. of the enzymatic extract were added to 6 ml. of the buffered solution covering the range pH 3.2 to pH 7.4. To the 8 ml. of the active mixture were then added (at time 0) 2 ml. of IAA solution ($50 \mu\text{g}/\text{ml}.$). In Figure 7 are presented the results showing that the destruction of IAA by the enzyme exhibits a sharp pH maximum (lag period as well as rate of inactivation) in the region of 5.7 to 6.4. Consequently and in confirmation of our previous data (Pilet 1957 b), we use pH 6.1 for the study of the IAA destroying enzyme.

Temperature

The effect of various temperatures on rate of enzymatic destruction of IAA was only examined in four experiments. Results are summarised in

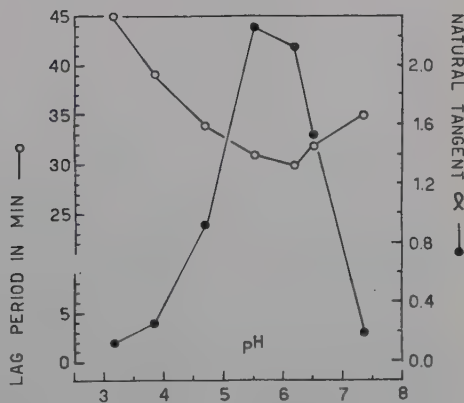


Figure 7. Action of the pH on the IAA destruction. Active mixture: 2 ml. (5 mg. fw/ml.) — buffered solution ($\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$): 6 ml. — IAA solution: 2 ml. ($50 \mu\text{g}/\text{ml}.$).

Table 4. *Lag period and tangent α of the activation curves of the enzymatic IAA destruction in relation to the temperature of the incubation.* 2 ml. of enzymatic extract (5 mg. fw/ml.), 6 ml. of buffered solution (pH: c. 1), 2 ml. of IAA (50 μ g/ml.).

Temperature in °C	Lag period in min.	Natural tangent α
12.5 \pm 1.5	35.7 \pm 3.7	0.04
16.4 \pm 1.3	32.6 \pm 3.0	0.51
22.7 \pm 1.4	31.5 \pm 2.5	1.17
31.3 \pm 1.0	25.4 \pm 2.6	2.71

Table 4 and it can be concluded that the lag period decreases with increasing temperature while the rate of inactivation of IAA increases if temperature increases.

Conclusion

The study of IAA destruction due to the action of various enzymatic systems implies precise and exact conditions for analysis. In fact, the concentration of the active extract, as well as that of the IAA solution used, plays an essential role in the kinetics of IAA inactivation. However, other factors interfere, amongst which the role played by pH and temperature should be stressed. The method of treatment suggested in this paper makes it easy to analyse the mode of action of certain effectors: H_2O_2 , 2,4-dichlorophenol and gibberellic acid. Yet, interpretation of the three phases of the enzyme action remains difficult as it is well known that destruction of IAA does not give only one product of oxidation (see Manning and Galston 1955, Galston 1956, Ray 1958, Pilet 1961 d). A rapid examination of the oxidation products is, in conclusion, necessary. The high activity of an IAA oxidising enzyme, obtained from the root tip of *Lens*, has made possible (Pilet 1960 a and b, Pilet and Lerch 1961) the characterisation of the oxidation products of the enzyme system.

By the chromatographic separation¹ and with the root tip extracts, four different substances were detected, using IAA labeled with C^{14} :²

Product I (Rf: 0.83) which is certainly β -indolylaldehyde as we previously demonstrated (Pilet 1960 a and b, and 1961 d); (see also Racusen 1956).

Product II (Rf: 0.18) and *Product III* (Rf: 0.30) which are not indolic compounds. It is possible, according to Manning and Galston (1955), that

¹ Descending chromatography ($25^\circ\text{C} \pm 0.5$) was used (Pilet 1958): Whatmann 1 — isopropanol (80)/28 % ammonia (10)/deionized water (10).

² The substance used is β -indolylacetic C^{14} α acid synthesised according to the technique of Pichat, Audinot, and Monnet (1954); specific activity of this product is 6.9 mc/mM.

these substances are all the same neighbour compounds of *o*-formamido-acetophenone and *o*-amino-acetophenone.

Product IV (Rf: 0.69) which was recently detected (Pilet 1961 d) with high concentrated root tip extract. This compound seems to be very close to the 3-hydroxy,3-methyloxindole observed with *Omphalia* enzyme by Ray and Thimann (1955, 1956), Ray (1956). *Product IV* gives the Tollen aldehyde reaction.

Consequently, (see Figure 8) the following scheme can be proposed in which it can be seen that IAA destruction occurs by a parallel transformation of the side chain and indole ring.

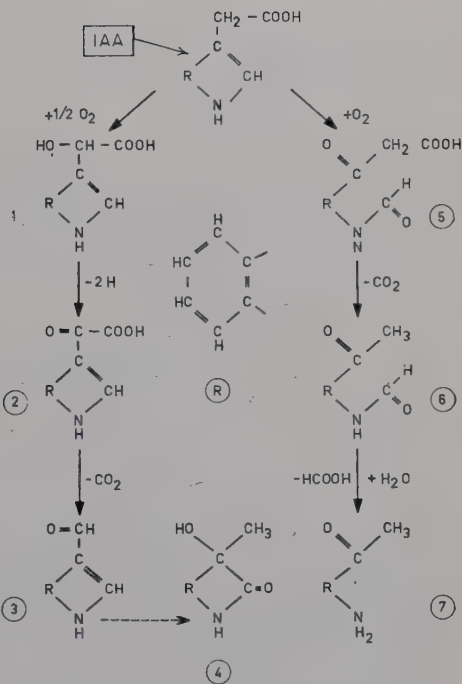


Figure 8. Possible ways of the IAA destruction.

1. β -indolyl-glycolic acid
2. β -indolyl-glyoxylic acid
3. β -indolylaldehyde (*Product I*)
4. 3-hydroxy, 3-methyloxindole (*Product IV*)
5. *o*-formamido-benzoyl acetic acid
6. *o*-formamido-acetophenone and
7. *o*-amino-acetophenone (similar to the *Products II* and *III*).

Summary

1. IAA destruction by root cells extracts following three distinct phases: a lag period, an activation phase and an equilibrium state.
2. The induction phase and the natural tangent, which is measured on the curve giving the IAA inactivation at the middle of the steady phase, permit a characterisation of the kinetics of the auxin catabolism.

3. Results of the experiments were then analysed in terms of enzyme bio-kinetics.
4. Relations between enzyme activity and enzyme concentration and substrate level are examined. As an oxidating agent H_2O_2 was used and the action of 2,4-dichlorophenol and gibberellic acid on the IAA inactivation was studied.
5. Analyses of pH and temperature effects were then made.
6. Finally, the importance of the various oxidation products resulting from the IAA destruction by the root tip extracts is discussed.

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Responses of Pea Roots to Growth Substances

By

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In experiments carried out to study the effects on root growth of growth substances applied to the hypocotyl of pea seedlings it was found that 2,4-dichlorophenoxyacetic acid (2,4-D) had a considerably stronger inhibitory activity than 3-indolylacetic acid (IAA) (Eliasson 1959). While as little as 0.1 μ g. 2,4-D gave a persistent inhibition of root growth the inhibition caused by 1 μ g. IAA was of only short duration. This difference in response has been subjected to further studies comprising also a number of other growth regulators. The experiments described demonstrate that there are marked divergencies in the response of pea roots to phenoxy compounds on the one hand, and to IAA and, in some respects, also to other auxins known to have low phytotoxicity, on the other hand.

Methods and Results

Responses to substances applied to the hypocotyl

Pea seedlings (*Pisum sativum*, variety "Torsdagsärt III") were grown on filter paper, according to an earlier described technique (Eliasson 1959). Some more details of this technique will be added here. For germination of the seeds plastic boxes (13 \times 13 \times 17 cm.) with a grating of glass rods 10 cm. above the bottom were used. On the bottom of the boxes there was a layer of water a few centimeters deep and sheets of filter paper long enough to reach the water were hung over the glass rods. The seeds, which had been soaked in water overnight, were pressed in between the glass rods with the radicle pointing downwards. After germination for two days the seedlings were fitted with small glass tubes around the hypocotyl by means of rubber tubing and placed on moist filter paper in polyethene sacks as

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Table 1. *Growth response of pea roots to growth regulators applied to the hypocotyl.* The seedlings were mounted on moist filter paper in polyethylene sacks. After 24 hours 0.02 ml. buffer solution containing the substances in the quantities recorded was pipetted into glass tubes fixed around the hypocotyl. The length of the roots was then 40 to 50 mm. The growth of the roots in per cent of the control is recorded for the following periods: I: 0—6 hours; II: 6—24 hours; III: 24—48 hours after application of the substances. — For each test 10 seedlings were used. The values are the averages from 5 independent experiments for IAA, IBA, NAA, NOA, 2,4-D, and TIBA and from 3 or 4 for the other substances.

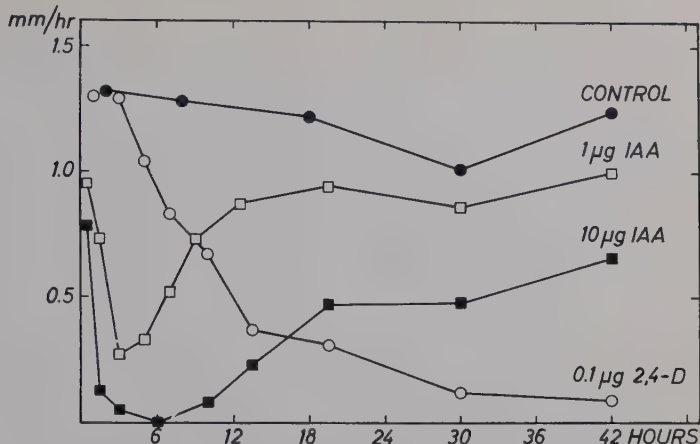
Substance	Root growth in per cent of the control								
	0.1 µg			1 µg			10 µg		
	I	II	III	I	II	III	I	II	III
3-indolylacetic acid (IAA)	100	98	114	59	92	110	16	63	101
3-indolylbutyric acid (IBA)				89	96	96	78	90	102
1-naphthylacetic acid (NAA)	84	93	118	64	72	91	44	36	84
2-naphthoxyacetic acid (NOA)				91	70	67	79	24	27
phenoxyacetic acid							91	74	90
2,4-dichlorophenoxyacetic acid (2,4-D)	95	62	49	90	9	2	51	0	0
2-methyl-4-chlorophenoxyacetic acid (MCPA)	99	58	58	83	2	0	45	0	0
4-chlorophenoxyacetic acid	98	84	51	82	15	1	63	0	0
3-chlorophenoxyacetic acid				69	70	97	55	11	61
2-chlorophenoxyacetic acid				84	78	93	90	83	105
2,4,5-trichlorophenoxyacetic acid (2,4,5-T) ...				112	93	98	88	48	63
2,4,6-trichlorophenoxyacetic acid (2,4,6-T) ...							92	82	85
phenylacetic acid				100	83	89	100	86	89
2,3,5-triiodobenzoic acid (TIBA)							80	83	104

described in the earlier paper. Between the seedlings were placed strips of filter paper. The function of these strips was to prevent the plastic film from sticking to the moist filter paper and thus obstructing a straight growth of the roots.

The growth substances were dissolved in phosphate buffer solution (1.5 mM KH_2PO_4 , 0.5 mM Na_2HPO_4 , pH 6.5). With this buffer solution the response to low 2,4-D quantities was somewhat weaker than that obtained in the earlier published experiments with a citrate-phosphate buffer solution. To ascertain that there was no leakage of solution from the hypocotyl tubes with the technique used, methylene blue solutions were applied in the tubes in some experiments. In no case was the colored substance observed to escape from the tubes.

Table 1 shows the results obtained in a series of test with various growth substances. Of these 2,4-D, MCPA and 4-chlorophenoxyacetic acid had a considerably stronger inhibitory effect than the other substances. The inhibition obtained with IAA, NAA and IBA was strongest during a period following rather soon after application of the substances. Later the roots recovered from the inhibition. A more detailed picture of the time course of growth is given by the curves in Figure 1. With the most active phenoxyacetic acids, on the other hand, the inhibition was slight during the first hours after application but increased progressively with time. It may be noted

Figure 1. The time course of root growth after application of IAA or 2,4-D to the hypocotyl. The substances were applied when the roots were 20 to 25 mm. long. Each curve is founded on two experiments, each with ten seedlings.



that the inhibition caused by NOA has a time course similar to that obtained with the phenoxy substances. This substance also has been reported to cause abnormal growth phenomena similar to those obtained with certain phenoxy compounds in tomato leaves (Zimmerman *et al.* 1952) and in cabbage seedling roots (Jagendorf *et al.* 1952). As pointed out earlier (Eliasson 1959), a characteristic response of the roots to the most active phenoxy substances is that the elongation stops a certain time after application of the substances. Subsequently the roots swell a few millimeters above the tip. IAA causes a similar swelling of the root during the period the root



Figure 2. Shadowgraphs of pea seedlings 24 hours after application of growth substances. From left to right: Control, 0.1 µg. 2,4-D, 1 µg. IAA.

elongation is inhibited. The typical appearance of the roots 24 hours after treatment with 2,4-D and IAA is shown by the shadowgraphs in Figure 2. The inhibition caused by 0.1 μ g. 2,4-D was considerably weaker in the experiments of Table 1 than in those of Figure 1 or in those earlier published (Eliasson 1959). The cause of this divergency was found in further experiments to be the difference in the length of the roots when the substances were applied. When seedlings with longer roots were used it was necessary to apply larger quantities of 2,4-D to obtain the same degree of inhibition.

The time course of root elongation on filter paper moistened with solutions of growth substances

The marked differences in time course of root growth after application of different auxins to the hypocotyl suggested investigation of the time course of root growth with growth substances added to the nutrient solution used for moistening the filter paper. When 2,4-D is applied to pea roots growing in flowing solution in narrow glass tubes a progressively stronger inhibition has been obtained (Audus 1949, Eliasson 1959). In these investigations evidence was also found that washing of the roots counteracted accumulation of 2,4-D in the tip region. If the roots are grown on filter paper the contact between the roots and the growth solution is rather limited. This may be expected to influence the absorption of externally applied growth substances into the roots but also the leakage of the same substances out from the roots.

Ten seedlings were grown in each polyethene sack with 50 ml. nutrient solution (pH 6.6) added. No glass tubes were used around the hypocotyl in these experiments. The seedlings were kept in position on the filter paper by means of elastic cords which were placed around the sack and the supporting masonite disk (cf. Eliasson 1959). To ascertain that the roots were brought in contact with the solution from the beginning of the experiment the sacks were placed in a nearly horizontal position for an hour immediately after the seedlings had been placed in them. During the remainder of the experimental period the sacks were placed in the usual manner with a deviation of about 20 degrees from the vertical plane, in which case the surplus solution was kept in the bottom of the sacks.

The results of one experimental series are given in Table 2. With 2,4-D, MCPA, 2,4,5-T, and NAA the inhibition increased during the test period. The response of the roots was, however, rather varying and some roots continued to grow after most of the roots in the same treatment had stopped growing. Thus the lower ones of the average values presented in Table 2 are the means of many non-growing and few growing roots. This great variation in growth response between the individual seedlings probably is due to the differences in contact between the seedlings and the filter paper. It must

Table 2. *The time course of root growth inhibition caused by growth substances to pea roots growing on filter paper. 50 ml. nutrient solution with growth substances added was used to each sack with ten seedlings. Average growth values are given in mm. for the control and in per cent of the control for the treatments.*

Substance	Concentration (M)	Number of roots	Time in hours from beginning of treatment			
			0 — 6	6 — 24	24 — 48	48 — 72
Control (mm.)	—	59	6.8	22.6	31.7	25.1
2,4-D	10^{-7}	19	94	91	58	33
	3×10^{-7}	19	84	54	12	2
	10^{-6}	20	66	22	3	0
MCPA	10^{-7}	18	94	61	21	10
	3×10^{-7}	20	85	40	4	0
	10^{-6}	20	40	17	0	0
2,4,5-T	10^{-6}	20	91	92	83	83
	3×10^{-6}	20	75	47	15	0
	10^{-5}	20	24	10	0	0
NAA	3×10^{-7}	19	59	60	21	16
	10^{-6}	19	41	31	10	3
	3×10^{-6}	20	25	13	4	0
IAA	10^{-7}	20	96	87	84	106
	10^{-6}	17	68	59	44	72
	10^{-5}	20	38	50	36	45
	10^{-4}	20	0	40	34	49

be pointed out that the time course of growth in the presence of phenoxy substances obtained is only applicable to pea roots. Wheat seedlings or rooted cuttings of aspen grown on filter paper with the same technique showed no time-dependent decrease in growth rate of the roots in presence of 2,4-D (unpublished experiments).

The inhibition pattern shown by IAA diverges strongly from that of the other substances. Rather varying results were obtained in different experiments. In general the inhibition at the beginning of the growth period was followed by a partial growth recovery. A comparison of the values of Table 2 with those obtained by Åberg and Jönsson (1955) shows that the inhibition obtained with IAA on filter paper is much weaker than that obtained when the roots are submerged in the test solution. The amount of IAA absorbed by roots growing on filter paper is probably too small to maintain an adequate internal concentration of IAA in the root tissue (cf. Åberg 1950). The small difference in growth rate between roots growing in 10^{-4} M and in 10^{-6} M IAA may be due to some adaptation mechanism (cf. Burström 1957, Audus and Bakhsh 1961), or to a rapid microbial decomposition of IAA in the higher concentrations.

Pea seedlings used as test plants for phenoxy compounds

The high sensitivity of pea seedlings to phenoxyacetic acids suggested the use of this plant for assaying synthetic growth substances in plant extracts.

The inhibition of root elongation is a sensitive but unspecific reaction to auxins which has been used in several test methods (see Linser and Kiermayer 1957). The technique utilizing the response of root growth to application of growth substances to the hypocotyl was found to be useful as an assay method with a great specificity for auxins of the phenoxy type. However, the labor involved led to efforts to modify the technique. In this work it was found that an assay method utilizing the over-all morphological effects caused by the tested substance rather than only root elongation would be more specific and even more simple than the usual root growth tests. For this reason growth conditions which permitted development of pronounced swellings and effects on lateral roots as responses to 2,4-D treatments were chosen for the growth of the test plant. As some experiments carried out with the test method further elucidate the great divergencies in the response of pea roots to phenoxy acetic acids and IAA, they will be briefly described in this paper.

The experimental material was pea seedlings germinated for two days in the way described on page 803. Selected seedlings were grown for five days in 10 ml. solution in test tubes (inner diameter 14 mm. length 125 mm.) in light from fluorescent lamps and with the temperature $25 \pm 1^\circ\text{C}$. The nutrient solution in which the growth substances were dissolved had the following composition: 1.5 mM KH_2PO_4 , 0.5 mM $(\text{NH}_4)_2\text{HPO}_4$, 1.0 mM $\text{Ca}(\text{NO}_3)_2$, 0.1 mM MgSO_4 , 0.01 mM MnCl_2 , 0.01 mM H_3BO_3 , 0.01 mM Fe-citrate. The seedling was kept in position in the test tube with only the root dipping into the solution by a ring made of rubber tubing which was placed immediately above the solution surface.

The shadowgraphs reproduced in Figure 3 show the typical responses obtained with 2,4-D and IAA. While IAA causes only weak responses in concentrations below 10 ppm., 2,4-D causes a marked inhibition of lateral root elongation already at a concentration of 0.01 ppm., i.e., 0.1 μg 2,4-D per seedling. Increasing concentrations of 2,4-D gives several other responses, viz., inhibition of root and shoot growth and swelling of the root combined with abundant forming of laterals. MCPA was found to be equally active as 2,4-D and to cause essentially the same responses; 2,4,5-T was about three times less active but caused the same responses as 2,4-D. NAA had weaker activity than the mentioned phenoxy compounds and caused partly other responses. From tests with other substances it may be mentioned that TIBA in concentrations from 1 to 30 ppm. strongly inhibited development of laterals while other aspects of growth were largely unaffected. Inhibition of the formation of laterals was also observed as a result of treatment with 2,4,6-T and MH (cf. Audus and Thresh 1956).

The growth effects induced by a certain concentration of 2,4-D. were very reproducible under the conditions used. When the method was used for



Figure 3. *Growth responses of pea seedlings to 2,4-D and IAA.* Each seedling has grown for five days in 10 ml. solution containing the substances. From left to right: Control, 0.01 ppm., 0.1 ppm., 1 ppm. and 10 ppm. 2,4-D; 0.1 ppm., 1 ppm., 10 ppm., and 100 ppm. IAA.

assaying phenoxy compounds, dilution series of the solution with unknown concentration of the substance were tested parallelly with series of known concentrations. Comparison of the plants in the two series at the end of the five-day test period mainly according to the principles used by Hitchcock and Zimmerman (1951) for tomato plants, made it possible to estimate the concentration of the unknown solution with a fair degree of accuracy. Südi *et al.* (1960) recently described an assay method which also utilizes the over-all morphological effects caused by growth regulators on seedlings and pointed out the merits of this technique.

Discussion

There is little reason to doubt that the growth inhibition and the swelling of the root tips after application of phenoxy-type auxins to the hypocotyl are due to the fact that the active substances are actually translocated to the growing region of the root. Although merely the fact that root growth is inhibited cannot be claimed to prove that there is really a translocation of the active substance to the root tip, it may be strong evidence in favor of such a conclusion (cf. Behrens and Morton 1960). In many investigations it has been found that 2,4-D and related substances are translocated with the

assimilate stream in the phloem. Using C^{14} -labelled 2,4-D Fang (1958) found that this substance was effectively translocated from the leaves to the roots of pea. Further the similarities of the swellings developed after application of the substances directly to the root and those developed after application of the substances only to the hypocotyl strongly indicate that the active substance moves to the root tip. In recent investigations retention of phenoxy substances by tissues along the translocation path has been found to be an important phenomenon affecting translocation of these substances (Crafts and Yamaguchi 1958, 1960, Canny and Markus 1960). Such a retention may explain the fact that in the present experiments the response of the roots to small amounts of 2,4-D applied to the hypocotyl was dependent on the length of the roots. These experiments also provide evidence that other auxins like IAA, NAA and IBA or some growth-active conversion products of them are rapidly translocated from the hypocotyl to the growing tips. The fact that Thimann (1936) did not obtain any inhibition of root growth of pea seedlings when he applied IAA to the cut stumps of the shoots may be due to a deficient translocation of the substance from this organ into the roots.

The experiments demonstrate that, while the effects of relatively high doses of IAA are largely overcome by the plants, the effects of 2,4-D and related substances grow progressively stronger even when only small quantities of the substances are applied. This may be due partly to a more rapid inactivation of IAA than of phenoxy compounds. It has been found that IAA but not 2,4-D is rapidly inactivated in pea tissues by conjugation with aspartic acid (Andreae and Good 1957) or by oxidative decarboxylation (Fang *et al.* 1959). Besides, it is probable that a contributing cause of the more severe effects in phenoxy substances is the power of these substances to induce disturbances in cell processes not caused by IAA. Some examples of such disturbances reported in the literature may be mentioned. Bond (1948) found 2,4,5-T to inhibit maturation of vascular tissues in pea roots, while Torrey (1953) reported IAA to accelerate the maturation of primary xylem elements. Further, 2,4-D has been reported to cause accumulation of growth inhibitory substances in plant tissues (Fulfs and Johnsson 1950, Key and Galitz 1959), to inhibit the polar transport of IAA (Hay 1956, Niedergang-Kaimen and Leopold 1959), and to decrease the rate of decarboxylation of IAA (Fang and Butts 1957).

Summary

The responses of pea seedlings roots to various growth substances were studied. The substances were applied in three ways:

1. To the hypocotyl of seedlings growing on moist filter paper in polyethylene sacks.

2. To the solution used for moistening the filter paper with the same culture method as in 1.

3. To the culture solution of seedlings growing in test tubes. With this method mainly the effect of growth-regulators on other aspects of growth than root elongation was studied. The method is thought to be suitable as an assay method for phenoxy-type auxins.

With all three methods pronounced differences were obtained in the response of the seedlings to phenoxyacetic acids with auxin activity on the one hand and IAA on the other. When the substance were applied to the hypocotyl, the elongation of the roots was inhibited by smaller quantities of the phenoxy auxins than of IAA. Furthermore, the inhibition caused by IAA was followed by rapid growth recovery while phenoxy compounds caused a more permanent inhibition. The effects of NAA and IBA were similar to those of IAA. The root growth of seedlings grown on filter paper moistened with phenoxy auxins in concentrations giving only moderate growth inhibition in the beginning of the experiment decreased progressively with time and most roots stopped growing in a day or two. Also when the seedlings were grown in 10 ml. nutrient solution in test tubes the effects of added phenoxy auxins were enhanced with time.

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Studies in the Physiology of the Lichen *Collema*

IV. The Occurrence of Polysaccharides and some Vitamins outside the Cells of the Phycobiont, *Nostoc* sp.

By

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The physiology of the lichens is still only little investigated, and we know very little about the laws which regulate the balance between the alga and the fungus and ensure that one of the partners does not wholly prevail over the other in the lichen thallus. The physiology of both the lichen partners can vary very much from species to species, and it is therefore probable that the balance can be maintained in different ways in different lichens.

The *Collema* lichens have no typical haustoria and often grow on substrata very poor in soluble organic substances. It is therefore of interest to investigate the extracellular occurrence of organic nutrient materials formed by the algal cells, *i.e.*, in these investigations, substances in the filtered medium in which the phycobiont, *Nostoc* sp., has been growing. It has previously been shown that the phycobiont produces extracellular nitrogenous substances (Henriksson 1951, 1957) and also substances which inhibit the growth of the mycobiont, *Collema tenax* (Sw.) Ach., em. Degel. (Henriksson 1960). However, on an agar medium, made from the filtered algal medium, it is possible to keep the mycobiont in culture, but with poor growth (Henriksson 1958).

In this paper is reported the occurrence of polysaccharides and some vitamins of the B-complex in the medium in which the phycobiont has grown.

To obtain the algal filtrate, the same culture conditions and the same methods were used in these investigations as in those previously described by the author.

Liberation of polysaccharides

Fogg (1952) reported that polysaccharides, in addition to nitrogenous substances, occurred in the medium in which *Anabaena cylindrica* had grown. It has also previously been shown that some marine and fresh water flagellates liberate polysaccharides to the culture medium (Allen 1955, Lewin 1955, 1956, Guillard and Wangersky 1958).

In these investigations of the culture medium of *Nostoc*, using the method described by Lewin, it was found that this algal species also had the power to liberate polysaccharides. A white stringy precipitate of polysaccharides was formed in ethanol. This precipitate could be collected in a fine strainer and dried. Table 1 shows the amount of the precipitate and the algal growth in 10 day-old cultures, in which the alga was grown both with and without sodium nitrate in the nutrient solution. The result is reminiscent of the production of extracellular nitrogenous substances, previously reported (Henriksson 1957). It was shown that a relatively high concentration of combined nitrogen was liberated to the medium when the alga was grown in a nutrient solution without nitrate, but with sodium nitrate in the medium no liberation of combined nitrogen took place under certain conditions. Further investigations of the polysaccharide production showed, however, that the polysaccharides seems to be liberated eruptively into the medium in aging cultures. Even with heavy inoculum the polysaccharides are seldom found in the algal filtrate before 30 days growth, both in cultures with and without nitrate in the medium. The results in Table 1 are thus not typical for the extracellular polysaccharide production of this species. The medium in very old cultures may become so viscous as a result of the high content of polysaccharides that it is impossible to filter it through filter paper. In contrast to the results obtained by Lewin (1956) for extracellular polysaccharide production by *Chlamydomonas parvula*, the polysaccharides liberated by this *Nostoc* species do not seem to parallel cell multiplication. The results are more comparable to those reported by Guillard and Wangersky (1958) for other species of flagellates, where polysaccharide liberation began at the

Table 1. Yields of polysaccharides, not purified, from different media in which the alga had grown for 10 days,

		Dry weight of alga in mg.	Dry weight of the polysaccharide precipitate per mg. dry weight of alga
Bristol's modified medium	Without nitrate	17.9 \pm 3.4 (7)	0.94 \pm 0.01
	With nitrate	21.7 \pm 3.0 (8)	No precipitate

Table 2. *Approximative amounts of polysaccharides and proteins in the medium in which the alga had grown for 4-5 weeks.*

Experiment	Polysaccharides in mg. per 250 ml.	Proteins in mg. per 250 ml.
I	6.9 ± 1.8 (3)	6.7 ± 0.3 (3)
II	8.7 ± 1.3 (3)	11.5 ± 0.5 (3)

end of the logarithmic phase of growth and increased during the stationary phase or senescence of the cultures.

The precipitates of polysaccharides were shown to contain about 34 % inorganic salts. An approximate value for the crude ash-free polysaccharide can thus be calculated. Also the approximate content of the proteins in the filtrate can be calculated by multiplying the amount of nitrogen, obtained from Kjeldahl analyses, by the factor 6.25. Table 2 shows the values obtained in this way, for two different experiments. The alga had grown in Fernbach flasks in 500 ml. nutrient solution without nitrate, and the filtrate was divided into two parts, one for each analysis. The values show that the liberation of proteins and polysaccharides is of the same magnitude in these cultures, but the differences between the two experiments sustain the view that the liberation of polysaccharides is independent of that of proteins.

Vitamins in the medium in which the phycobiont has been growing

To determine whether there are vitamins outside the algal cells, which might be utilized by other microorganisms, analyses for various vitamins belonging to the B-complex were performed. The analyses were made using microbiological assay. Thiamine was estimated by using *Phycomyces Blakesleeanus*, according to Schopfer and Jung (1937), and the other vitamins by using *Lactobacillus arabinosus*, according to Barton-Wright (1952). The results are shown in Table 3. The assay values are for solutions in which the alga had grown for 4 weeks. If the alga had grown for a shorter time (2-3 weeks) before filtering, either there were no detectable amounts of vitamins

Table 3. *The amount of extracellular vitamins from 4 week-old cultures, expressed per mg. dry weight of alga.*

Thiamine	0.010 — 0.015 µg
Riboflavin	trace
Nicotinic acid	0.1 µg
Pantothenic acid	0.020 — 0.025 µg
Biotin	0.0006 µg

or the amounts were less per mg dry weight of alga than for filtrates from 4 week-old cultures. On the other hand, in filtrates from cultures of 10 weeks, where an appreciable autolysis had set in, the values were somewhat higher.

No major difference could be found in the amounts of vitamins in the filtrate from cultures which had fixed molecular nitrogen and those which had assimilated nitrate. However, the nitrate-assimilating cultures tended to liberate the vitamins later than the nitrogen-fixing ones.

In addition to the vitamins analysed there are certainly other growth-substances in the medium in which this *Nostoc* species has grown. It is also probable that to a large extent these substances are to be found on and in the mucilago-sheaths of the alga. The amounts of the vitamins available for the microorganisms which live in the immediate neighbourhood of the alga would then be greater than is indicated by the analysis figures.

Discussion

Because of the occurrence of polypeptides, polysaccharides and vitamins outside the cells of this symbiotic *Nostoc* species, the immediate surroundings of the algal cells will be a special environment, which may be favourable for other microorganisms. However, such microorganisms are also subjected to antibiotic substances excreted by the alga (Jacob 1954, 1957, Henriksson 1960). It is probable that the mycobiont of *Collema* is specially adapted to this environment. As has previously been shown this fungus has a lethal effect on living cells of the phycobiont and in this way it may also obtain its nutrient requirements (Henriksson 1958). The extracellular products can also be of great importance since they can form chemical complexes with other dissolved substances (Fogg and Westlake 1955).

Summary

In the medium in which the phycobiont (*Nostoc* sp.) of the lichen *Collema tenax* had grown, polysaccharides and vitamins belonging to the B-complex can occur. These substances are particularly to be found in aging cultures.

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Effect of Anisomycin on Growth and Respiration of *Candida*

By

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Anisomycin, an antibiotic produced by some *Streptomyces* species, was originally discovered as an antiprotozoan agent (Sobin *et al.* 1954), and primarily used clinically as such. Its antifungal properties were, however, first demonstrated by Lynch *et al.* (1954), who tested the effect of anisomycin *in vitro* on several dermatophytes and fungi causing systemic mycosis. It proved to be effective only against *Candida*, *C. albicans* being the sole species tested. Anisomycin has been used clinically against candidosis. It also inhibits the growth of *Saccaromyces cerevisiae*, and such a strain is recommended as a test organism in the bioassay of anisomycin (Grove *et al.* 1955). Furthermore, it has been tested as a possible preservative for fresh vegetables (Goodman *et al.* 1957).

In the present investigation, we have tested the effect of anisomycin on the growth and respiration of various *Candida* species.

Material and Methods

Eight different strains of *Candida*, representing 7 species, i.e., *Candida albicans* (1/59), *C. krusei* (81/59 and 200/59), *C. parapsilopsis* (188/59), *C. pseudotropicalis* (115/59), *C. pulcherrima* (BF 22/59), *C. robusta* (BF 20/59), and *C. tropicalis* (26/59) were kindly placed at our disposal by Dr Å. Frisk, Stockholms Stads Bakteriologiska Laboratorium, Stockholm, and used in the present investigation. *Saccaromyces cerevisiae* ATCC 9763 was also used.

The fungi were grown for 24 hours on slant cultures of Sabouraud Dextrose Agar (Difco) containing 1 per cent (w/v) neopeptone, 4 per cent glucose, and 1.5 per cent Bacto-Agar. The cells from a slant culture were transferred in 10 ml. of saline solution into 500 ml. of Wickerham's synthetic medium (Wickerham 1951) with

0.5 per cent glucose in a Fernbach flask, and incubated for 12 hours at 28°C on a reciprocal shaker. These cultures had an optical density of about 1.0 at 610 mμ, when measured with the uninoculated medium as a blank in a Beckman B spectrophotometer. After centrifuging twice in saline solution, the cells representing late log phase cells were suspended in water, generally to a concentration of 6×10^7 /ml., and controlled by counts in a haematocytometer (Helber). The total nitrogen content of 6×10^7 cells was found to be about 0.05 mg.

Growth determinations were made turbidimetrically in tubes in a Beckman B spectrophotometer at 610 mμ.

Respiratory experiments were performed in the conventional Warburg apparatus at 30°C. The following quantities were used: 0.5 ml. of cell suspension, 2 ml. of 1/15 M Sørensen NaK-phosphate buffer, pH 6.0, various amounts of substrate and anisomycin, 0.2 ml. of 10 per cent KOH (in centre well) and distilled water to a total volume of 3.2 ml. per vessel.

Subcellular fractions were prepared by sonic disruption in the following way. Late log phase cells, about 2 g. wet weight, were suspended in 10 ml. of cooled saline solution and treated for 45 minutes in an MSE ultrasonicator (Model 60 W) with the vibrator probe, diam. 3/4", at 1.55–1.60 amp. audio frequency. The gas phase was argon, and the temperature was maintained at about 2°C. About 75 per cent of the cells were disrupted. The suspension was repeatedly centrifuged at 4000 g, until the supernatant was free from all whole cells and cell walls, which was checked in the phase-contrast microscope.

Protoplasts were prepared with the enzyme preparation "Suc digestif d'Helix pomatia", L'Industrie Biologique Francaise, as recommended by Bachmann and Bonner (1959). Isolation of protoplasts was performed with cells from an 18-hour shaking culture and enzyme treatment for 5 hours, as suggested by Eddy and Williamson (1957) in their studies of protoplasts from *Saccaromyces* species. It was also performed according to Marini *et al.* (1961), using 4-hour cultures and treatment for 3–4 hours at 28°C with 10 per cent snail enzyme, 20 per cent saccharose in a 0.03 M Sørensen phosphate buffer, pH 5.8, and addition of 0.1 ml. of a 1 per cent aqueous cysteine-HCl solution to each ml. of enzyme. The effect of cell wall destruction was followed in the phase-contrast microscope. When about 50 per cent of the cells were transformed to protoplasts, differentiating, repeated, cautious centrifuging was started. A phosphate buffer, pH 5.8, with 0.82 M mannitol was used for washings and as a suspending medium. It was, however, found difficult to obtain 100 per cent protoplasts; consequently, some unaffected cells and disrupted protoplasts were presumably present in the preparations.

The anisomycin, a crystalline monobasic substance with the formula $C_{14}H_{19}NO_4$, was kindly supplied by the Pfizer Co. It was dissolved in minimum amounts of methanol, and diluted in water. The amount of methanol did not influence the results.

Results and Discussion

Effect of anisomycin on growth. The effect of anisomycin on the growth of 7 species of *Candida*, at a concentration of 0, 12.5, 25, 50, and 100 μg/ml. of liquid medium, respectively, was tested in tubes with Sabouraud liquid medium. Inoculation was made with 1 ml. of cell suspension with a density

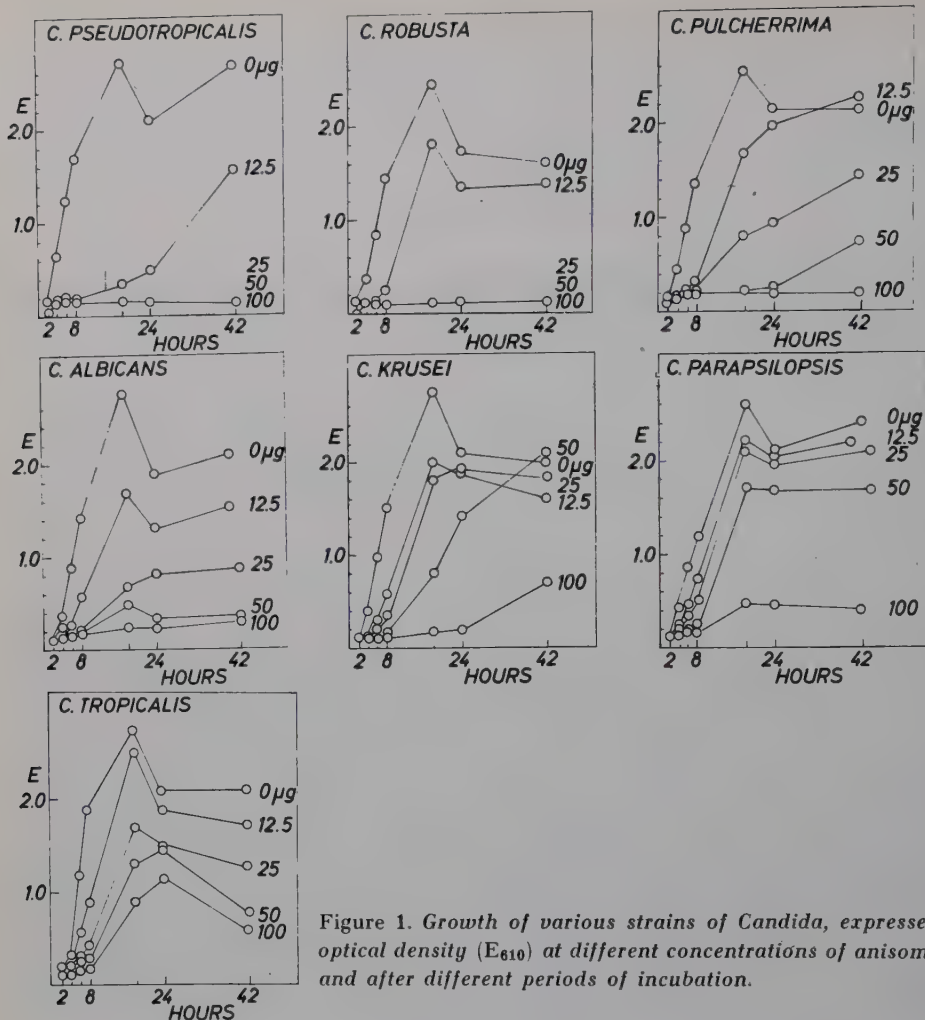


Figure 1. Growth of various strains of *Candida*, expressed as optical density (E_{610}) at different concentrations of anisomycin and after different periods of incubation.

of about 10^7 cells, giving a total volume of 10 ml., and an optical density of about 0.05. The inoculated tubes were shaken at 30°C in a water bath, and readings were made after several periods. In Figure 1, the different species are arranged after falling sensitivity to anisomycin, *Candida pseudotropicalis* and *C. robusta* being the most sensitive. 25 $\mu\text{g}/\text{ml}$. inhibited their growth completely during the whole test period, whereas 100 $\mu\text{g}/\text{ml}$. were required to produce the same effect on *C. pulcherrima*. In all the other species, even the highest concentration tested brought about only a lengthening of the lag phase. The maximum growth in series with any addition of anisomycin did

Table 1. *Effect of anisomycin at a concentration of 200 and 400 µg/ml., respectively, on the oxidation of glucose by different strains of Candida and by Saccharomyces cerevisiae. Oxidation measured in µl O₂ uptake during 60 minutes, and expressed as a percentage of control values. 10⁷ cells per ml.*

Species	O ₂ uptake in µl.				O ₂ uptake %	
	Endo- genous	Total			Anisomycin	
		Anisomycin				
		0	200 µg/ml	400 µg/ml	200 µg/ml	400 µg/ml
<i>C. pulcherrima</i>	7	40.5	18.5	20	46	47
<i>C. parapsilopsis</i>	7.5	41	24	23	58	55.5
<i>C. pseudotropicalis</i>	5	82	54	43.5	66	57
<i>C. robusta</i>	8.5	70.5	51	44	69.5	62.5
<i>C. albicans</i>	10	37	29	25	80.5	68.5
<i>C. tropicalis</i>	14	31.5	25	21.5	82.5	69
<i>C. krusei</i> 200/59	9.5	52.5	45.5	42.5	86	81.5
<i>C. krusei</i> 81/59	—	43	40.5	49	95	111
<i>Saccharomyces cerevisiae</i>	—	51	37	37	75.5	74.5

not, however, amount to the corresponding maximum control values. Analogue tests in Wickerham's synthetic medium gave principally the same results. All strains of *Candida albicans* tested by Lynch *et al.* (1954) were more sensitive to anisomycin than any of ours. They mentioned a minimum inhibitory concentration of 1.5–12.5 µg/ml. after an incubation time of 18 or 24 hours.

Respiratory experiments. The effect of anisomycin on glucose oxidation by the species of *Candida* used above, as well as by *Saccharomyces cerevisiae*, was studied. Cells from the late log phase were used. Anisomycin was added, giving a concentration of 200 and 400 µg/ml. The O₂ uptake was measured by the Warburg direct method. The data are given in Table 1, where the *Candida* species tested are listed in the order of falling degree of sensitivity. The reaction ranged from complete insensitivity to addition of anisomycin, *e.g.* *C. krusei* 81/59, to a decrease in O₂ uptake of about 50 per cent, *e.g.* *C. pulcherrima* and *C. parapsilopsis*, and 35 per cent in the case of *C. pseudotropicalis*. Of these three species showing a reasonable decrease in O₂ uptake, *C. pseudotropicalis* was that most strongly inhibited in the growth test. The decrease in O₂ uptake may reflect either an inhibition of respiratory enzymes, or a fungicidal effect, decreasing the number of respiring cells.

Tests for fungicidal action were performed with *C. pseudotropicalis*. Late log phase cells in the same number/ml. as in the Warburg experiment *i.e.* 10⁷ cells/ml., were incubated in shaking tubes in a water bath at 30°C with 200 and 400 µg/ml. of anisomycin for 0, 30, 60, and 120 minutes, respectively. After each time, samples were diluted, plated and counted after 42 hours. A

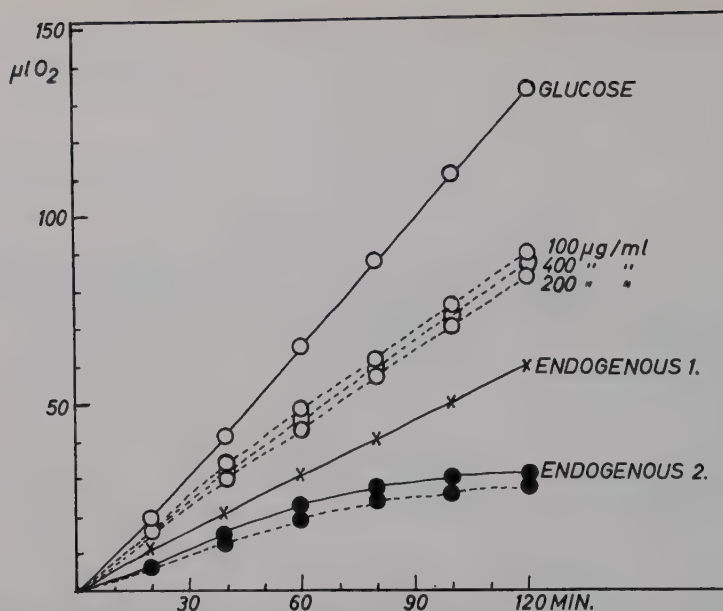


Figure 2. Effect of three concentrations of anisomycin on O_2 uptake on glucose of *Candida pseudotropicalis*. 0.2 mM glucose added, and anisomycin in the side arm, tipped 20 minutes after the first reading. Final concentration of cells 10^7 /ml., and of anisomycin 100, 200, and 400 μ g/ml. Gas phase: air.

— without anisomycin

--- with anisomycin

decrease in the number of colony-forming cells was, in fact, found during the first period, whereas the tests at 60 and 120 minutes gave more or less equal numbers. Thus, at the concentrations used, the decrease in O_2 uptake was primarily caused by the fungicidal effect on *C. pseudotropicalis*. Even if the basic reason for the decrease in O_2 uptake was not an inhibition of respiratory enzymes, the changes in O_2 uptake may give some information about the fungicidal mechanism. *C. pseudotropicalis* was used as test organism.

When this investigation was started, the endogenous respiration was always very low (See Table 1 for representative values). As far as could be ascertained, it was unaffected by anisomycin. The organism had, however, changed to a high endogenous respiration, as can be seen in Figure 2, where the values for the endogenous respiration of two different experiments (1 and 2) are given.

The endogenous respiration was almost unaffected by anisomycin, and the same relative effect by anisomycin on the total O_2 uptake was observed.

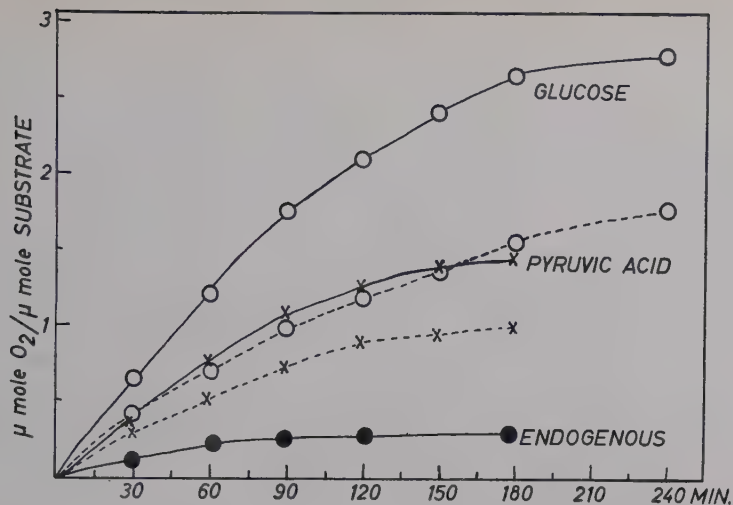


Figure 3. Effect of anisomycin on O_2 uptake on glucose and puruvic acid of *Candida pseudotropicalis*. 2 μ mol glucose, 2 μ mol puruvic acid, respectively, and anisomycin in the side arm, tipped at 0 minute. The final concentration of cells $2 \cdot 10^7$ /ml., and of anisomycin 200 μ g/ml. Gas phase: air.

— without anisomycin

--- with anisomycin

In all experiments, the relative effect increased with time, *e.g.* giving at 200 μ g/ml. a decrease in O_2 uptake of 12.5, 28, 34, 35, 36.5, and 37.5 per cent after an incubation time of 20, 40, 60, 80, 100, and 120 minutes, respectively. A comparison with the plate count experiment shows that the levelling off in numbers of colonyforming cells and in percentage O_2 uptake started at the same time *i.e.*, after 60 minutes.

The relative effect of anisomycin on the O_2 uptake was about the same either glucose, puruvic, or citric acid was used as substrate. Figure 3 gives data for the O_2 uptake in μ mole per μ mole glucose or puruvic acid.

Since many antibiotics exert their bactericidal or fungicidal effects (Newton 1958, Henis *et al.* 1960) by damaging the cell membrane, it seemed interesting to compare the effect on the O_2 uptake by whole cells and protoplasts of *C. pseudotropicalis*. Finally, it appeared of interest to test the O_2 uptake of a subcellular fraction of the same fungus, in which the intact cell membrane had been removed. The results are given in Figure 4 and Table 2. The final relative decrease, measured on whole cells and protoplasts, was about the same with glucose as substrate. During the first 60 minutes, the relative effect on the protoplasts was small, probably depending on some disturbances in them immediately after the snail enzyme treatment. After 60 minutes, the decrease was of the same value as in the series with whole cells. In the sub-

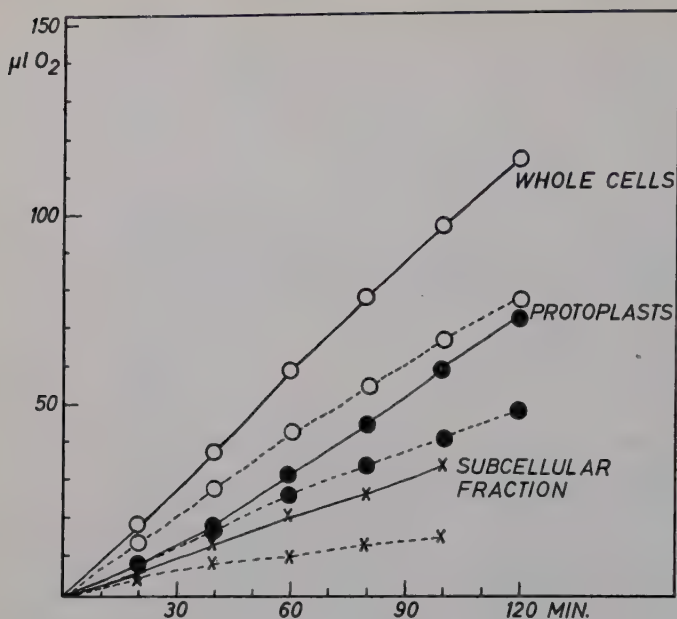


Figure 4. Effect of anisomycin on O_2 uptake by whole cells, protoplasts and subcellular fractions of *Candida pseudotropicalis* 0.2 mM glucose added, and anisomycin in the side arm, tipped 20 minutes after the first reading. Final concentration of cells 10^7 /ml., and of anisomycin 400 μ g/ml. Gas phase: air.

— without anisomycin
 ---- with anisomycin

cellular fraction, the decrease in O_2 uptake was greater during the whole test period than that of whole cells or of protoplasts. The inhibition increased with time. Thus, the relative anisomycin effect on O_2 uptake was evidently increased after removal of the lipid-containing cell membrane, which must be attributed to an effect on the respiratory enzymes, or the sites of respiratory enzymes. Furthermore, the effect increased with time from 32 to 58 per cent. It seems attractive to tentatively explain the increase in inhibition of the subcellular fraction with time as depending on damage to the lipid-containing respiring particles. The effect of anisomycin on the sediment from the subcellular fraction, obtained after centrifugation at 25,000 g, is under investigation.

Table 2. Decrease in glucose oxidation by whole cells, protoplasts, and subcellular fraction after various test periods at an anisomycin concentration of 400 μ g/ml. Decrease expressed as a percentage of the corresponding values for glucose oxidation without anisomycin.

Time minutes	Whole cells	Protoplasts	Subcellular fraction
20	22	0	32
40	27	7	42
60	29	17.5	52.5
80	31	25	52
100	32	31.5	58
120	33	34	—

Summary

1. The minimum growth-inhibitory concentration of anisomycin, determined after an incubation time of 42 hours, and with a cell density of 10^6 cells/ml. at zero time, is found to be 25 $\mu\text{g/ml.}$ in the case of *C. pseudotropicalis* and *C. robusta*, but more than 100 $\mu\text{g/ml.}$ in all other strains tested.
2. Anisomycin is found to decrease the respiration. At a concentration of 200 $\mu\text{g/ml.}$ and with glucose as substrate at pH 6.0 the decrease amounts to 50 per cent of that of the controls in the case of *C. pulcherrima* and *C. parapsilopsis*, to about 35 per cent in *C. pseudotropicalis*, and less in all other species.
3. The relative effect of anisomycin on the O_2 uptake by whole cells, protoplasts, and a subcellular fraction has been measured.

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The Effect of the Water Deficit on the Stomatal Movements in a Carbon Dioxide-Free Atmosphere

By

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The present study is a continuation of the experiments concerning the action of carbon dioxide on hydroactive closure of the stomatal cells, described in earlier papers (Stålfelt 1957, 1959). These earlier experiments showed that CO_2 promotes hydroactive closure, and thus has an inhibitory effect on the photoactive opening reaction, since this is in mobile equilibrium with the hydroactive closing reaction.

In my subsequent work, my aim was to determine the dependence of the CO_2 -sensitive opening movements on the size and duration of the water deficit, as well as the size of the passive component which, at a suboptimal deficit, generally forms part of the opening movement. For lack of a better term, I have in the following denoted the opening movements that are caused by removal of CO_2 as *the CO_2 -sensitive movements*.

The experimental plants were chiefly *Vicia Faba* and *Ranunculus ficaria*.

Measurement of the width of the guard cells was performed microscopically, in the same way as described earlier (Stålfelt 1959, p. 692). The cells were measured on rectangular pieces of leaf, 5×10 mm in size.

The CO_2 of the air in the object chambers was removed by KOH. The object chambers consisted of glass cuvettes (volume 1 litre) that could be closed by rubber stoppers. The method is a modification of that used by Linsbauer (1917) to demonstrate the effect of CO_2 pressure on the opening and closing movements of the stomatal cells. Some hours before starting the experiment, a few ml of KOH solution were poured into each cuvette. The leaves were either put in tubes containing water, or placed on a metal net fitted into the cuvette.

Leaves enclosed in such a chamber continue to transpire, since the vapor tension on the surface of the leaf is greater than that over the KOH solution and close to

the walls of the cuvette. Transpiration is increased when sampling takes place, and when the leaves are removed for weighing. Owing to transpiration, a water deficit of varying degree arises in the leaves without water supply. One of the objects of the present experiments was to study the effect of this water deficit.

The water deficit of the leaves was calculated as the difference between the current weight of the sample and its weight on water saturation (when the experiment was started), and expressed as a percentage of the latter value. The leaves were therefore severed, and placed in water in a moisture-saturated chamber (a glass vat), where they were kept in darkness for 12 to 14 hours before the experiment. The leaves are water-saturated after a few hours. However, as pointed out earlier, the saturation weight is not constant, since the leaf continues to take up water later (Stålfelt 1955). Even after 40 hours, young leaves of *Vicia Faba* have not reached a constant weight. This has been interpreted by Čatský (1959) as a result of growth processes, which are associated with an uptake of water.

The water deficit was measured by weighing the leaves, in the same way as in my earlier experiments (1955). If transpiration — which is a result of treatment of the object as described above — does not lead to the desired deficit, the deficit can be regulated by altering the transpiration, *e.g.* by changing the moisture of the air around the object, or letting the object float on the water. It can also be regulated by changing the water supply (increasing it by renewing the cut surface on the leaf stalk, or decreasing it by depriving the sample of water).

Experimental

A. Dependence of the CO₂-sensitive movements on the water deficit of the leaves

Example of the experiments on the importance of the water deficit for the appearance and development of the CO₂-sensitive movements is given in Figures 1–3 and their accompanying legends. The experimental objects consisted of whole leaves, as well as leaves cut in pieces, that could be studied separately. The objects were placed in darkness. The CO₂ of the air was removed by KOH.

The experiments involve certain technical difficulties. For example, if whole leaves are used, and samples are taken twice an hour from the same leaf, it is impossible to avoid exposing the leaf to the CO₂ of the air, and to increased transpiration. These disturbing changes can be partly or wholly avoided by using several leaves, and enclosing each of them in a separate cuvette, samples being taken from each leaf only once an hour, or, *e.g.* once every other hour. Unfortunately, this procedure introduces the variation dependent on the difference between such factors as the age and development of the leaves. It is, however, possible to avoid this variation as well, by using only one leaf, but dividing it into small parts, each of which is placed in a single cuvette, and then studied separately. A drawback is that this limits the number of measurements.

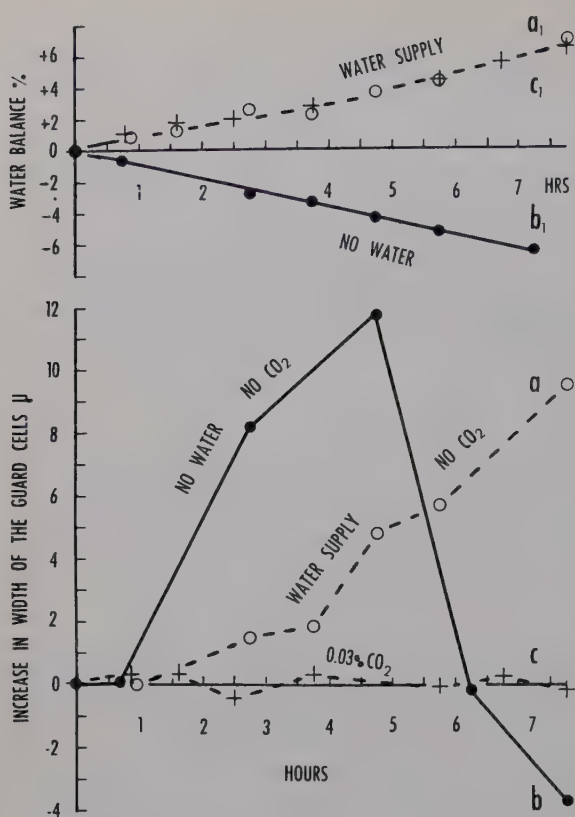


Figure 1. CO_2 -sensitive opening movements (stomatal movements elicited by removal of the CO_2 in the surrounding air) and their dependence on the water deficit of the leaf. — Object. *Vicia Faba* grown in the open; leaf with 2 leaflets; whole leaflets were used. Pre-treatment. Severed leaves were kept in darkness and saturated moisture for 12 hours. Width of guard cells at beginning of experiment (*a* and *b*): 30.5 and 30.7 μ respectively. Experimental conditions. Darkness, 20–22°C.

Experiment *a* and *a₁*. The leaf was placed with its stalk in water in a cuvette (1 liter), of which the floor was covered by a solution of KOH, and the opening closed by a rubber stopper. At each sampling, the leaf was weighed, and water balance (*a₁*) calculated (increase and decrease, respectively, in % of the weight of the water-saturated object). Experiment *b* and *b₁*. Leaf without water-supply during the experiment; treatment otherwise as in *a*. Experiment *c* and *c₁*. Leaf placed with its stalk

in water, and enclosed in a glass cuvette containing air with a normal CO_2 content; treatment otherwise as in *a*. — The experiment show that the stomata open in darkness when CO_2 is removed; that opening is interrupted and succeeded by closure (*b*) when the water deficit (*b₁*) of the leaf has reached about 4 %; and that the water output of the guard cells (*b*) does not cease with a decrease in their width to the initial value, but continues, so that the cell width becomes less than that of the control leaf (*c*). It also shows that CO_2 -sensitive opening is dependent on the pressure of the leaf tissue. Thus, in *a*, opening occurs under resistance from the turgor pressure of the adjacent cells; in *b*, this pressure is released as soon as the deficit arises and develops. The opening process is a result of interaction between passive and CO_2 -sensitive components.

In the course of the work, the latter method was found to result in the smallest variation in the values. It nevertheless requires relatively large leaves, and could not therefore always be used.

It is evident from the results that the CO_2 -sensitive effect — *i.e.*, the stomatal reaction elicited by removal of the CO_2 impediment — is dependent

Figure 2. Aim of experiment as in Figure 1. Object. *Vicia Faba*; leaf with 2 leaflets. Each leaflet was cut in 4 pieces and weighed; each piece was put into a separate cuvette containing KOH solution, and the opening closed by a rubber stopper. In 4 of the cuvettes, the pieces of leaf were in contact with the water (a); in the others, no water was supplied (b). For measurement of stomatal width and water balance, a sample was taken from each piece once only, or at 2-hour intervals. Otherwise as in Figure 1. — The experiment shows that pieces of a leaf react in the same way as a whole leaf; when the water deficit has reached about 4 % the opening movement is interrupted, and is succeeded by closure.

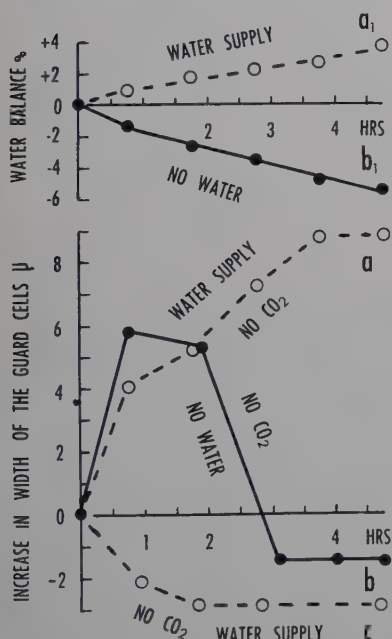
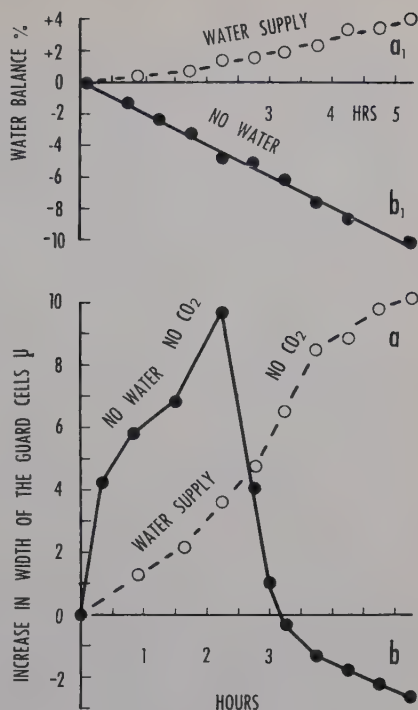


Figure 3. Experiment as in Figure 2, but supplemented by an object (c) in which transpiration was partly inhibited by letting the leaf float on water, with its upper side upwards. The dish with the leaf was placed in a cuvette containing KOH, and closed by a rubber stopper. In contrast to objects a and b, c could transpire from one side only, and water could be taken up not only through the stalk, but also through an incision in the leaf surface. Otherwise as in Figure 1. — A strong turgor pressure compresses the guard cells, and prevents CO₂-sensitive opening (c). Objects a, b and c show that the guard cells can react in three different ways after removal of CO₂. Which way is chosen depends on the hydratur of the object and its changes.

in the individual case on the hydratur of the object. In those cases in which transpiration and water uptake are so balanced that only a slight deficit develops, stomatal opening may proceed for several hours and reach high values. As soon as the deficit exceeds a certain, critical value (threshold value), opening is — on the contrary — discontinued, and is succeeded by closure. Finally, the opening movement may be completely lacking if no deficit occurs, or if the turgor pressure rises instead; in the latter case, a decrease in the width of the guard cells may sometimes take place (passive closure).

The dependence of the CO_2 -sensitive reaction on the water deficit is nothing essentially new. The light-induced opening reaction shows the same dependence (*cf. e.g.* Stålfelt 1959).

B. Dependence of the CO_2 -sensitive opening on the passive movements of the guard cells

Like the photoactive opening movements, the CO_2 -sensitive movements are inhibited by the turgor pressure of the leaf-tissue. If the pressure is high, opening cannot occur (Figure 3 c). Only when the leaf lost water, so that the pressure on the guard cells is decreased, is it possible for the cells to dilate and open. If the turgor of the guard cells is sufficiently high, opening already takes place initially, when the pressure from the epidermal cells decrease owing to the increasing transpiration. To this passive movement is added the CO_2 -sensitive movement, when the object is freed from CO_2 . Thus, the CO_2 -sensitive movement — like the photoactive one — has a passive companion. Its size varies, depending on variations in the actual turgor of the guard cells. The size of the passive part of the movement can be determined by measuring the passive movement in, for example, half of one leaf, and the size of opening in a CO_2 -free medium in its other half. Examples of such measurements are given in Figure 4.

In Figure 4, the hydroactive reaction is sufficiently strong, even at a deficit of 2 per cent, to interrupt passive opening in the case where the CO_2 of the air is present, whereas this does not apply in a CO_2 -free medium. If CO_2 is removed, the impediment to water uptake constituted by it disappears — according to Mouravieff (1956), starch also disappears and the osmotic value rises — water is taken up, and opening continues. Only with a deficit of about 4 per cent is the hydroactive reaction sufficiently strong to interrupt the CO_2 -sensitive opening as well.

The hydroactive reaction initiates or increases an output of water from the guard cells. According to earlier experiments, CO_2 partakes in this process in such a way that it increases the water output, *i.e.*, CO_2 potentiates

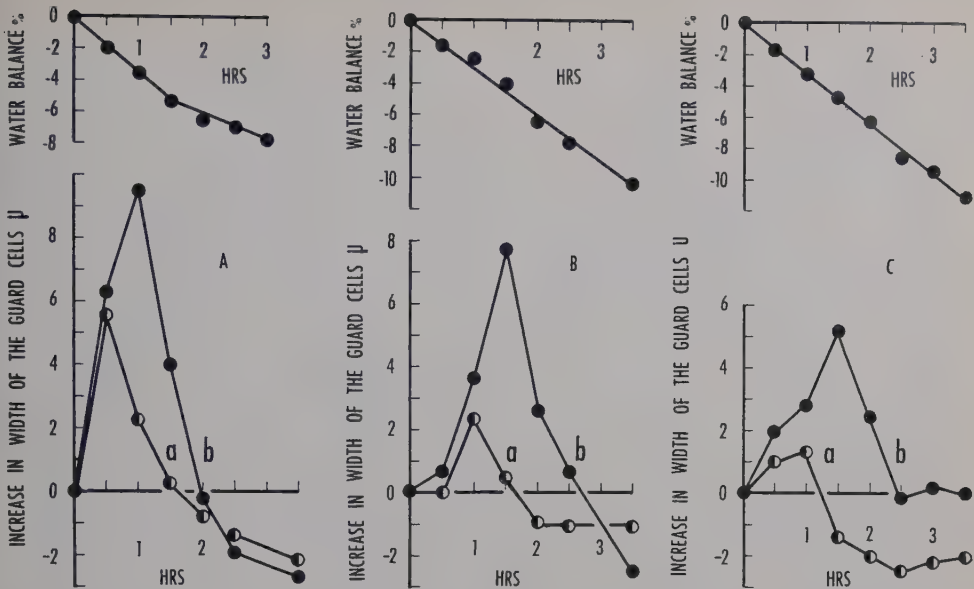


Figure 4. *Interaction between the passive and the CO_2 -sensitive opening.* Object. Leaflets of *Vicia Faba*. *Experiment A.* A leaf was cut in half; one half (*b*) was placed in a cuvette containing KOH solution, and fitted with a metal net on which the leaf rested. The other half (*a*) was placed in the same way in a cuvette containing KCl solution and air with a normal CO_2 content. At each sampling, the object was weighed, and the deficit calculated. Pretreatment and other conditions as in Figure 1. *Experiments B and C* of same kind as *A*. — Passive opening (*a*) continues until the object has reached a water deficit at which the hydroactive closing reaction is sufficiently strong to elicit closing. Opening proceeds for longer in CO_2 -free medium (*b*) than in CO_2 -containing; opening is interrupted and succeeded by hydroactive closure only when a higher deficit (4–5 %) is reached. As shown earlier, hydroactive closure is promoted by CO_2 (cf. Stålfelt 1959). Consequently, in the present experiment, closure starts at a lower deficit in *a* than in *b*; CO_2 lowers the threshold value of the water deficit.

the hydroactive effect (Stålfelt 1959). It is possible that the same CO_2 mechanism is active in CO_2 -sensitive opening as well, so that its removal decreases the water output or increases the water uptake.

C. Threshold value of the water deficit

In special experiments, I determined the threshold value of the water deficit, *i.e.*, the deficit at which CO_2 -sensitive opening is interrupted, and stomatal closure starts. Examples are given in Figure 5. They show that the threshold value of *Vicia Faba* lies in the vicinity of 3 per cent, *i.e.*, approximately the same value as that found to apply for stomatal closure in light

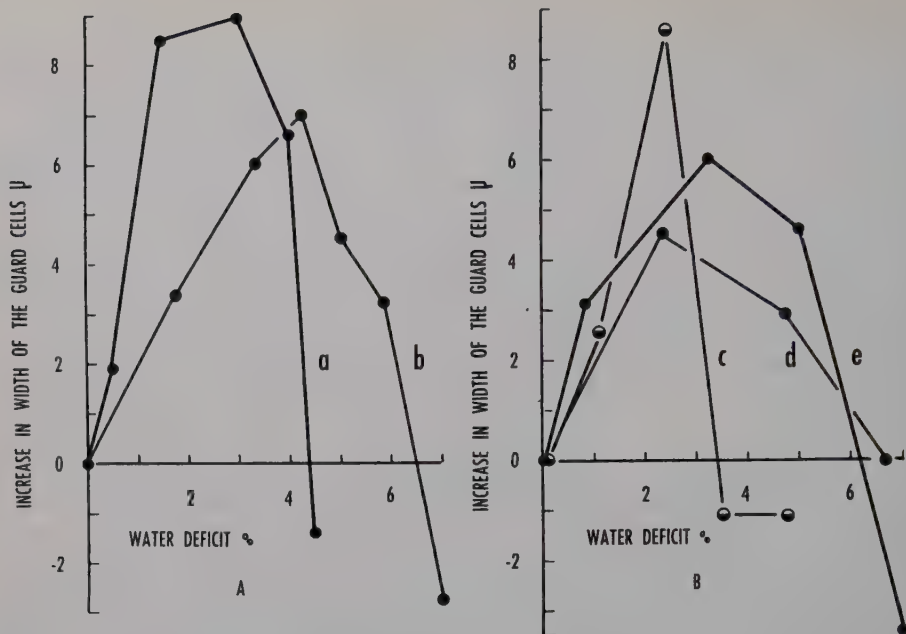


Figure 5. *Threshold value of the water deficit on CO_2 -sensitive opening. Object. Vicia Faba. a and c, whole leaflets; b, d and e, pieces of leaflet each put into a separate cuvette. Experiment otherwise as in Figure 1 b. — CO_2 -sensitive opening is interrupted by hydroactive closure at a water deficit (abscissa) of the order of magnitude of 2–4 ‰.*

(Stålfelt 1929, 1955). The photoactive and the CO_2 -sensitive opening processes thus show the same sensitivity to the water deficit. It must, however, be emphasized that a comparison of this kind is unreliable, since it presupposes agreement not only with respect to the kind of experimental object, but also with respect to age and previous history (*cf.* Pisek and Winkler 1953). Like other physiologic threshold values, that of the water deficit is probably dependent on the tonus and hardness of the object at the time of the determination, these — in turn — being determined by its previous history and environment. The factor that is, in the first place, decisive for the reaction of the guard cells to the water deficit is, presumably, the deficit of the surrounding cells. However, this deficit is in turn dependent on the water status of the leaf and of the whole plant, and changes with it.

D. *Dependence of hydroactive closure on the size and duration of the water deficit*

Figure 6 is an example of the effect of water deficits of the same duration but of varying size. The hydroactive effect increases with the size of the

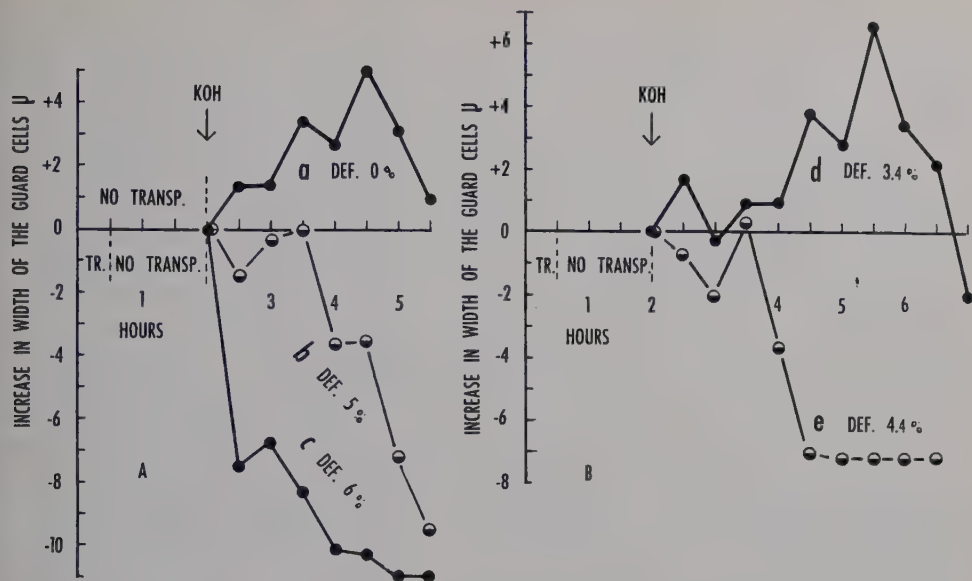


Figure 6. Relation between the size of the water deficit and the strength of the hydroactive reaction. *Object.* Leaves of *Ranunculus ficaria*. *Pretreatment.* Severed leaves were kept in darkness in a moisture-saturated chamber for 12 hours. Despite this, the stomata had an opening width of about $3\ \mu$. *Experimental conditions.* Darkness, $20-22^{\circ}\text{C}$. — *Experiment A.* Two leaves (*b* and *c*) were removed for a few minutes (*TR*) from the moist chamber, and allowed to transpire without a supply of water until their deficit was 5% (*b*) and 6% (*c*), respectively. The leaves were then returned (without water supply) to the water-saturated chamber. After 90 minutes (*NO TRANSP*) the leaves were transferred, still without water supply, to cuvettes containing KOH. For comparison, a third leaf (*a*) which had not undergone any water loss (*DEF. 0%*) was included: it was also put (without water supply) into a cuvette containing KOH. Owing to the deficit which arose, stomatal opening was interrupted in *a* after $2\frac{1}{2}$ hours. *Experiment B* is of the same kind as *A*, but the deficit is 3.4 and 4.4%. — The hydroactive closing process not only prevents CO_2 -sensitive opening (*b*, *c*, *e*), but also causes an output of water from the guard cells, so that their width becomes less than before the experiment.

deficit, and does not appear until a certain value (threshold value) is exceeded. In experiment *B*, this value lies between 3.4 and 4.4 per cent.

Figure 7 illustrates the effect of deficits of the same size but of varying duration. Closure does not start until a certain time — the reaction time — has elapsed. In the example in question, this time lies between 60 and 90 minutes.

It is evident from the experiments that CO_2 -sensitive opening and photoactive opening are in conformity in several respects. Thus, the water deficit

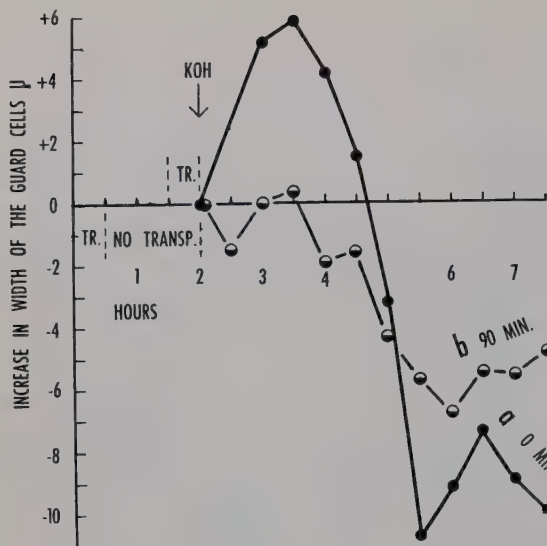


Figure 7. Relation between the duration of action of the water deficit and the onset and strength of the hydroactive reaction. Object, pre-treatment and experimental conditions as in Figure 6. — The water-saturated leaf (*b*) was exposed for a few minutes (*TR*) to transpiration, to produce a deficit of 5 %. It was then placed for 90 minutes (*NO TRANSP*) in a moisture-saturated chamber, but without water supply. After this, the leaf was transferred to a cuvette containing KOH. Another leaf (*a*) was treated in the same way, except that the deficit was produced immediately before exposing the leaf to KOH (*TR*). Here, (*a*) the time required for the deficit to elicit the hydroactive closing reaction is 60–90 minutes. During this time (the reaction time), CO₂-sensitive opening can take place, whereas this is not the case afterwards, when the closing reaction has started (*b*).

has the same threshold value, the reaction time is about the same, and the hydroactive effect increases with the size and duration of the deficit. This conformity indicates that the CO₂-sensitive opening — like the photoactive — is in equilibrium with hydroactive closure.

E. Conclusions

Experiments with *Vicia Faba* and *Ranunculus ficaria* show that the CO₂-sensitive opening movements of the stomata — like the photoactive opening movements — are dependent on the hydratur of the object. Thus, both kinds of opening movement are inhibited by high turgor of the leaves and are promoted by a slight water deficit; they are interrupted by the hydroactive closing movement when the water deficit of the object has reached a value of 2 to 4 per cent, and in both cases the hydroactive closing movement increases in strength with the size and duration of the deficit.

This conformity between the CO₂-sensitive and the photoactive movements lends support to the view that the CO₂-sensitive effect contributes to photoactive opening. That this effect alone is responsible for opening is an assumption which disregards the fact that photosynthesis acts concurrently with the CO₂-sensitive opening reaction. Thus, when CO₂ is utilized, photosynthetic products are formed, and *vice versa*. The osmotic value and turgor of the guard cells are altered by both effects.

Naturally, other effects of light and carbon dioxide are conceivable. (1) A change takes place in the pH of the cell sap and plasma (Sayre 1926, Freudenberg 1941). (2) It cannot be ruled out that CO₂ influences the permeability and the communication with subsidiary cells. Schaefer's (1956) investigations show that a change in CO₂ pressure (respiratory CO₂ and CO₂ of the air) in the chloroplast-containing roots of *Lemna minor* disturbs the contact between plasma and cell wall, so that the adhesion of the protoplasm to the cell membrane is altered. (3) Whether light acts in some other way than by photosynthesis is a controversial question. In any event, light has a thermic effect, since it vaporizes water in the transpiring leaf, and thus contributes to an increase in the water deficit. Such an effect can be reduced by filters of various kinds, but cannot be inhibited. Recently it was analysed in a thorough investigation by Kuiper (1961).

Since the two processes — the CO₂-sensitive and the photosynthetic — cannot take place independently of each other, it seems most likely that they interact. Photoactive opening would, in this case, be elicited by the following two kinds of process: (1) A decrease in CO₂ pressure, leading to a decreased or inhibited water output from the guard cells (*e.g.* depending on an increase in the quantity of osmotically active substance, *i.e.*, a transformation effect). (2) An increase in the guard cells' content of osmotically active substance, due to their own photosynthesis, *i.e.*, a production effect.

Such a reaction system for opening is fundamentally in agreement with the known facts regarding the closing movements of the stomata. There is, in fact, reason to believe that the closing movements are also dependent on an interaction between two kinds of process (*cf.* Williams 1954, Stålfelt 1957). They are: (1) A rapidly occurring decrease in turgor in the guard cells; it is caused by a water deficit and potentiated by CO₂, and is probably of an adenoid nature. (2) A slower reaction of osmotic nature, *i.e.*, a decrease in the cell content of osmotically active substance, this reaction also being initiated by a water deficit. — In this reaction system, the closing process, also in its components, appears as a reversible form of the photic opening process.

Both reaction systems — that of opening and of closure — are dependent in the individual case on the water deficit of the object. This also applies to the passive movements. Consequently, the factor primarily decisive for the opening width and movements of the stomata is neither light nor the CO₂ effect, but the water deficit of the object — or, more exactly, the hydro-active closing reaction. This is because the strength of this reaction is determined by the size and duration of the water deficit.

Discussion

Milthorpe and Spencer (1957), using the porometer method, studied the relation between the water content of the leaf, transpiration and resistance to air flow in the leaf, but were unable to distinguish the hydroactive reaction from the passive. Consequently, they were also unable to determine the threshold value of the water deficit at which hydroactive closure is initiated. Referring to these studies, Heath (1959) stated that the attempts to separate, on a basis of whole-leaf water deficit, "passive" and "hydroactive" phases of stomatal movements seem foredoomed to failure.

The reason why Heath came to this conclusion is that he compared experimental results from objects in different physiologic states. He did, in fact, compare the following kinds of experimental results (denoted here as 1 and 2).

1. Experimental results from objects with *small water deficits*, i.e., such that do not produce wilting — if by this term is meant that state occurring when the object has lost its turgor. In this part of the deficit gradient, the stomatal value changes with the deficit, and thus becomes a regulator of water metabolism. The regulator prevents wilting, as long as the water uptake is at least as large as transpiration when the stomata are closed. This group of investigations includes those that I have made, to which Heath refers in his comparison.

Moreover, in such investigations, the object has been allowed to transpire in normal air, and transpiration has taken place by normal diffusion from intercellular spaces and stomata. Finally, the water content of the object has been calculated in relation to the weight of the leaf, determined when it had been in darkness and a moisture-saturated chamber for 12 hours, with its stalk in water.

2. Experimental results from objects with *large water deficits*, i.e., wilting objects, or objects that had wilted just previously. The object did not transpire in air with normal moisture, but in air dried by calcium chloride and phosphorus pentoxide. During the porometer determinations, this air was sucked through the intercellular spaces of the leaf, the water transport from the leaf then being changed from diffusion to mass transport, with a flow of dry air. The moisture in the intercellular spaces then changed instantaneously from relatively high values to extremely low ones.

Such differences in the experimental conditions are responsible for differences in the results. The stomatal mechanism, like that of other organs, is dependent on the current state of the plant. It cannot work in the same way when the water deficits are large and not completely reversible, as when they are small and reversible. In any event, this applies to the hydroactive closing reaction, since it does not function normally with a high water deficit, but

is disturbed by incipient destructive processes, and by the movements of cells and tissues that are a result of the great variations in hydratur. To throw light on this question, it is necessary to make a closer study of the work on which Heath (1959) based his statement, namely, that of Milthorpe and Spencer (1957).

The experimental objects used by Milthorpe and Spencer consisted of leaves with both high and low water deficits. A study of the experiments with low water deficits discloses nothing that can be used to substantiate the view that the hydroactive reaction was not in action, or did not elicit stomatal closure at certain deficit. It is evident from their Figures 1-3 that the transpiration and porometer values changed after the water supply had been stopped or restored. Both the transpiration and porometer values can be used as more or less reliable indices of stomatal width. Both are indirect. Moreover, the porometer values include other resistance than that of the stomata, even if this is often disregarded, and such terms as "stomatal resistance" or "stomatal opening" are used instead of leaf resistance. If transpiration is taken as an index of the stomatal condition, their Figures 1-3 show that stomatal closure started after transpiration had continued for some time after interruption of the water supply. In Figure 1 (*Pelargonium*), this time amounts to 50-75 minutes, and in Figures 2-3 (wheat) to 30-50 and 30-40 minutes, respectively.

It is not, however, on the experiments with low water deficits that Milthorpe and Spencer chiefly based their discussion and conclusions, but on experiments which produced wilting. The leaves were exposed to cycles of wilting and recovery. It was found that after a period of wilting, the recovery of the water content of the leaf was less than that of the leaf in its pre-wilting condition, and also that after recovery of the leaf from a period of wilting, the stomata only opened partially. It was sometimes found that the stomata did not close or remain closed, but opened when another cycle of wilting was done. As far as the latter form of stomatal reaction is concerned, it can be pointed out that it is detrimental to the plant, and that the plant would lack the prerequisites for surviving a dry period if such a mode of reaction were to be usual one. Consequently, opening of the stomata on wilting must be interpreted as a sign that the stomatal cells have been injured by the water loss, or have been brought into such a condition that they can no longer function normally.

The facts that the leaves cannot completely regain their turgor, and that the stomata are unable to open to the same degree as formerly, also indicate that an inhibitory or disturbing event has taken place. It may be in the form of a reduction in the photic sensitivity of the guard cells of the kind I have demonstrated earlier (1955). Or it may consist of a drying out and collapse

of the cells closest to the intercellular spaces, thus exposing them to a destructive water deficit. A mesophyll cell, which has a diameter of only some tens of thousandths of a millimetre, and a cell membrane — whose thickness is only a small percentage of this value — may perhaps become dehydrated even after a few seconds when the humidity of the air in the intercellular spaces disappears, and is replaced by extremely dry, flowing air. Nor do we know how, in the individual object, such changes in volume influence the resistance of moving air, which is measured by the porometer.

The range of variation in the moisture of the air in the intercellular system of the leaf is unknown. Bange (1953) stated that the air in the intercellular spaces cannot be moisture-saturated, owing to the suction of the cell walls, but that the difference is inappreciable. The difference varies with the humidity of the external atmosphere. According to Klemm's (1956) calculations, the relative humidity in the intercellular spaces can fall to *e.g.* 90 per cent, if the humidity of the external air is 50 per cent. If the relative humidity is less than 100 per cent, it is — as pointed out by Klemm, and also by Slavik (1958) — lowest close to the stomatal apertures, and highest at the mesophyll cells, furthest in the intercellular canals.

However, irrespective of whether the value is 100 per cent or a few units less, the water loss of the mesophyll cells will be of another order of magnitude when the water transport through the intercellular spaces changes suddenly from diffusion to a rapid mass transport of dry air. Even if the vapor pressure deficit in the intercellular space were as large as 10–15 per cent — as the extreme cases in Klemm's calculations suggest — the suction force of the air in them would be more than 10 times greater when dry air is sucked in. If the deficit were nil or only a few per cent — which, presumably, is usually the case in turgescient leaves — the suction force would reach values hundredfold greater in the moment when the air changed to a dry one. The increase would be greatest in those parts of the leaf where the air enters. When such radical changes occur, it seems inevitable for the mesophyll cells to lose rapidly such a large proportion of their water content that the risk of a lethal deficit is imminent. It is true that the water loss caused by the porometer determinations is inappreciable if it is given in absolute figures. It can scarcely have any noteworthy influence on the total water content of the leaf. But the loss initially involves only those cells — among them the stomatal cells — adjacent to the intercellular spaces, and in relation to the water content of these cells the loss is great. It is these cells that are chiefly threatened by a destructive water deficit. The submicroscopic film of cutin that may be present on the mesophyll walls can be expected to decrease, but not to prevent, such a detrimental effect. This also applies

to the decrease of the permeability which, according to Klemm (1956), is associated with incipient drying.

The water loss causes a decrease in volume of the cells, until they have lost their turgor pressure and start to wilt. The time required for this to occur depends on the elasticity of the cell walls, as demonstrated by Krasnosselsky-Maximow (1925), Oppenheimer (1930) and Clark and Levitt (1956), among others. If the water loss continues to increase, the cells become deformed.

In addition, there are changes caused by differences in the elastic stretch of the cell walls. The elasticity of the mesophyll cells is high; it was found by Clark and Levitt (1956) to be 25 per cent. The epidermal cells are more rigid (Oppenheimer 1930), but nevertheless contract sufficiently to make the otherwise smooth outer aspect corrugated (Fogg 1947). Since the decrease in volume is greater in the mesophyll than in the epidermis, a change takes place in the tension normally present between the tissues, and to which the guard cells are adjusted. Owing to this change, the guard cells are exposed to pressure and stretching that do not occur as long the tissues retain some of their turgor. The normal movements of the guard cells are impeded by these mechanical changes.

As Iljin (1922) found, the deficits with a rapid onset are the most injurious. The explanation is that the course of the hydroactive reaction requires a certain time. If the deficit has a rapid onset, the reaction cannot be completed in time, *i.e.*, the guard cells do not have time to close, and their osmotic value is not reduced before the wilting stage has occurred. If the guard cells still retain some of their turgor at this time, they dilate and open when the pressure from the epidermis lessens. Thus, instead of being closed, they remain open. Such cases of opening movements in wilting leaves have been known for a long time (*cf.* Stålfelt 1956).

As well as these disturbances, which are chiefly of an anatomic-mechanical nature, there are others, which involve the physiologic processes in the guard cells. Iljin (1922), who studied these changes at water deficits of varying size and duration, classified them as reversible and irreversible. He found that even after a slight wilting the stomatal values were lower than normally; thus a result of the same kind as that reported by Milthorpe and Spencer (1957). In addition, Iljin found that the disturbances are intensified with a greater degree of wilting. The stomatal cells "büssen entweder vollständig ihre Regulierbarkeit ein, oder die Regulation geht nur noch in sehr beschränktem Masse vor sich." (p. 619). After greater wilting, the ability of movement ceases completely and, in extreme cases, the cells die. Iljin (1922) found that in leaves that had undergone strong wilting, up to 60 per cent of the stomatal cells had died. Furthermore, it has recently been demon-

strated by Polster and Fuchs (1960) that an injurious water deficit limits the regulatory activity of the stomata.

Such changes in mechanical relations and physiologic processes upset the stomatal mechanism; the movements of the guard cells become abnormal, are hindered or prevented, and their active and passive reactions become confused. Consequently, in wilting leaves and those that have newly wilted, it is — as stated by Milthorpe and Spencer (p. 436) — extremely difficult to separate passive from active changes, and impossible to determine threshold values of the water deficit. It can be added that another reason, that makes it impossible to determine the threshold value of the water deficit for the hydroactive reaction in wilting leaves, or in those that have just wilted, is that threshold values of this kind occur only at small water deficits. This is because the hydroactive closing reaction is elicited by the water deficit before it has reached such a size that wilting occurs. This does, in fact, constitute its importance as a regulator of the plant's water state.

Unfortunately, the study of Milthorpe and Spencer's interesting work is made more difficult by the too meagre description of their methods, and of the ways in which the calculations were made. Although they refer to earlier papers (Gregory *et al.* 1950), these also fail to provide sufficiently detailed information. For example, the authors give the water content of the object as a percentage of the weight, but it is not clear on which weight value or which standard degree of turgor the calculation was based, nor in what condition the leaf was when the initial value was determined. The hydration of the leaf is characterized by such expressions as "maximum turgor" and "full turgidity", but it is not stated how these conditions were determined. It is said that values were obtained from experiments "in which the leaf water content was constant at maximum turgor . . ." (p. 420). One cannot but wonder how the authors arranged the experiment to achieve this state. For, a constant maximum turgor does not, as a rule, occur in transpiring leaves — least of all in severed leaves, and it was such that were studied. As soon as transpiration has started, a water deficit arises which, in its turn, causes the increase in suction force which initiates and maintains the water supply. Presumably, the only exceptions are such objects in which the root pressure is sufficiently strong to be alone responsible for this process.

In a discussion of the relation between the water content of the leaf and the transpiration, the presentation is in such a form that it gives the reader the possibility of interpreting the contents both as an argument in favour of the view that such a relation exists, and as an argument against this view. One of the conclusions reads: "Stomatal movement was found to exert a large controlling influence on the transpiration rate, whereas water content had an extremely small or negligible effect" (p. 413). According to another

conclusion, the stomata are dependent on the leaf water content: "However, considerable changes of stomatal aperture resulted from changes in leaf water content and these mask influences of the latter on transpiration" (p. 421). It does not seem clear how these conclusions are to be interpreted. If the leaf water content influences the stomata, and these influence transpiration, the leaf water content should be a primary prerequisite for transpiration. In this case, its effect cannot be denoted as "small or negligible". It may be that the authors are referring to components of the transpiration system other than the stomata. Unfortunately, they have made no distinction between these two kinds of components. The reader is apt to infer that "the aperture of the stomata is largely independent of the level of leaf water content". Heath (1959, p. 229) also drew this conclusion from Milthorpe and Spencer's presentation.

Summary

A study has been made of those opening movements of the stomatal cells elicited by removal of carbon dioxide — denoted here as the CO_2 -sensitive opening movements — and of the dependence of these movements on the hydratur of the object. The experimental objects were *Vicia Faba* and *Ranunculus ficaria*. It is found that in cases where transpiration and water uptake are so balanced that only a slight water deficit appears (2—5 per cent in the plants in question), the CO_2 -sensitive movements can proceed for several hours, and reach high values. Opening is, on the contrary, interrupted and is succeeded by hydroactive closure when the deficit has exceeded a certain critical value — the threshold value — and has a certain minimum duration — the reaction time. The hydroactive effect increases in strength with the size and duration of the deficit.

If no deficit forms, or if the turgor pressure is raised instead, the opening movement may fail to occur, or is replaced by a decrease in width of the guard cells, despite removal of CO_2 .

When the deficit starts to occur, a passive opening effect is sometimes added to the CO_2 effect.

The CO_2 -induced mode of reaction is in conformity with the mode of reaction of the guard cells to light. This conformity substantiates the view that the CO_2 effect contributes to stomatal opening. The assumption that it is the sole cause of opening is not compatible with the fact that it arises concurrently with, and as a result of, photosynthesis in the guard cells, associated with production of an osmotically active substance in them. Consequently, the CO_2 -sensitive effect and the current photosynthetic production are simultaneously active in photoactive opening.

Like other hitherto known kinds of stomatal movements, the CO_2 -sensitive effect is dependent on the hydratur of the object. The chief factor decisive for the state and movements of the stomata is neither light nor CO_2 , but the water deficit of the organ or, more exactly, the hydroactive closing reaction. This is because the strength of this reaction is determined by the size and duration of the water deficit.

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Effect of Ethylenediamine Tetraacetic Acid (EDTA) on Ion Uptake and Retention by the Protoplasmic Particulates

By

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It was previously demonstrated that Ca^{45} ions adsorbed on the cell wall of potato slices were available for uptake by intracellular particulates (Mertz and Levitt 1961). However, the fact that both the mitochondria and microsomes picked up activity made it impossible to determine whether one or both of the particulates captured the ions from the cell wall. Because the exchange of ions during the homogenization procedure may have in part been responsible for both particulates becoming labeled the following experiments were developed in order to determine to what extent ion exchange plays a part in the labeling of the mitochondria or microsomes.

Chasson (1959), using freeze dried tissue demonstrated that when EDTA was introduced with radiocalcium in the homogenization medium it effectively reduced the adsorption of Ca^{45} by the mitochondria and microsomes by 80 and 85 percent respectively. These data therefore suggested that a particular concentration of EDTA might be found which would prevent exchange of Ca^{45} during the homogenization procedure, and thus indicate which particulate is initially labeled during the metabolic uptake of ions.

Material and Methods

The methods were essentially as described previously (Mertz and Levitt 1961). In determining the extent of ion exchange during homogenization of prelabeled tissue, potato slices (Russet variety) 2 mm. thick were washed 48 hrs. in running tap water. The slices were then rotated for one hour at room temperature through 1700 ml.

distilled water containing $1.3 \times 10^{-6} M$ Ca^{45} (21 μc). The slices were then immediately placed in 250 meq./l. stable calcium at $2-3^\circ\text{C}$ for 5 min., followed by three successive 10 min. washes in distilled water. Following the final wash the tissue was homogenized in 100 ml. 0.25 M sucrose buffered at pH 8 with phosphate buffer containing various concentrations of tetrapotassium ethylenediamine tetraacetate (EDTA). Following fractionation of the tissue the mitochondria and microsomes were isolated and assayed for their activity (see Mertz and Levitt 1961).

In determining the effect of EDTA on ion retention by the protoplasmic particulates, the particulates were labeled as described above or an equivalent amount of Ca^{45} ($1.3 \times 10^{-6} M$) was added to the homogenization medium (100 ml.). Following fractionation of the tissue the particulates were isolated, suspended in an appropriate volume of 0.25 M sucrose buffered at pH 7.5 and separated into equal fractions. Each fraction was then pipetted into 25 ml. 0.25 M sucrose (pH 7.5) containing various concentrations of EDTA. The particulates were again isolated and their activity determined.

Nitrogen determinations of the mitochondrial and microsomal fractions were carried out according to the Nessler procedure of Umbreit *et al.* (1957).

Results

Figure 1 illustrates that concentrations of 0, 0.002 and 0.004 N EDTA had no effect upon the activity of the mitochondria while 0.008 N results in a 66 percent decrease in activity. The inability of EDTA at the lower concentrations to alter the mitochondrial activity suggests that either EDTA is effective in preventing ion exchange during the homogenization procedure

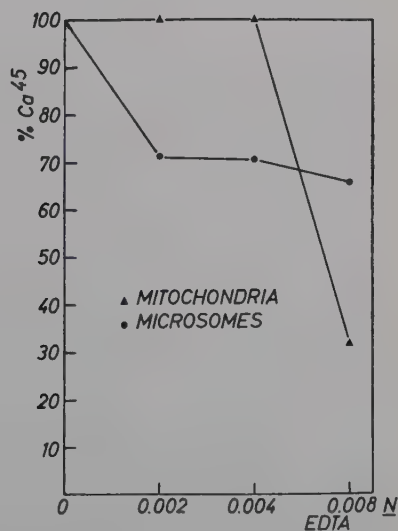


Figure 1. Effect of EDTA on ion retention by particulates during homogenization. On the abscissa concentration of EDTA N , on the ordinate retention of Ca^{45} %.

or that the relatively nonpolar lipid membranes of the mitochondria are unable to adsorb appreciable amounts of calcium. The ability of the mitochondria to give up their calcium at 0.008 *N* indicates that at higher concentrations, EDTA can penetrate the mitochondria and more effectively chelate calcium than the calcium binding sites of the mitochondria. This ability of the mitochondria to give up calcium is certainly one of the first requirement of a "carrier system" involving ion uptake as well as ion transport.

In contrast to the mitochondria the microsomes lose 28 percent of their activity in 0.002 *N*, but further increases of EDTA have very little effect on the microsomal activity. The rather easily removable calcium suggests that during the homogenization procedure some Ca^{45} is loosely adsorbed by the microsomes; however, the failure to remove Ca^{45} at increasing concentrations of EDTA suggests 1) that they are stronger chelaters of Ca than EDTA, and 2) that the major portion of calcium absorbed by the microsomes resulted during the preceding metabolic uptake. To what extent the microsomes participate in ion uptake remains to be further investigated; however, their inability to release their bound calcium would make them rather poor "carriers" in the metabolic uptake of ions.

Because the tissue itself would contribute a considerable number of ions during the homogenization procedure which would chelate with EDTA, thus rendering the initial concentrations of EDTA less effective toward Ca^{45} , labeled particulates were isolated, separated into equal fractions and suspended in various concentrations of EDTA. The particulates were again isolated and their activity determined (Table 1).

The results of Table 1 illustrate the increased effectiveness of EDTA. 0.002 *N* EDTA causes a 50 percent decrease in the mitochondrial activity. This loss of activity again emphasizes the ease by which the mitochondria can give up a portion of their bound calcium. The microsomes again exhibit their rather striking ability to retain their bound calcium.

Table 1. *Effect of EDTA on ion retention by particulates isolated from tissue prewashed 48 hr. prior to labeling. Counts per min. and mg. N.*

EDTA <i>N</i>	Mitochondria	Microsomes
Control	182	145
0.002	91	135
0.004	74	118
0.008	62	107
0.016	76	122
0.032	75	122

Mitochondria labeled for 1 hour at room temperature in 21 μC Ca^{45} Final molarity in 1700 ml. 1.3×10^{-6} *M*. Microsomes similarly labeled for 2 hours.

Table 2. *Effect of EDTA on ion retention by particulates labeled during the homogenization procedure. Counts per min. and mg. N.*

EDTA N	Mitochondria	Microsomes
Fresh tissue		
Control	33	26
0.002	25	28
0.004	16	18
0.008	19	29
0.016	17	24
0.032	12	27
Tissue washed 48 hr.		
Control	21	25
0.002	8	—
0.004	11	19
0.008	6	17
0.016	11	17
0.032	7	11

Uptake of Ca^{45} from the homogenization medium by the mitochondria and microsomes isolated from fresh and prewashed tissue. Equivalent amount of Ca^{45} used in Table 1 was added to 100 ml. of homogenization medium.

Several investigators (Sutcliffe 1952, Shean and Levitt 1959, and Laties 1959) have demonstrated that prewashing storage tissue for several hours enhances ion uptake. This enhancement has been considered to be caused by the production of carrier molecules which are capable of trapping ions. In an attempt to determine which of the intracellular particulates might contain the carriers, fresh and prewashed tissue were homogenized in the presence of Ca^{45} . Table 2 compares the activity of the particulates isolated from fresh and prewashed tissue. Although the data do not indicate which of the two particulates possess the carrier system, they do confirm the previous suggestion that ion exchange plays a rather insignificant role in the labeling of the mitochondria or microsomes during the homogenization procedure. A comparison of Tables 1 and 2 reveals that had ion exchange during the homogenization procedure been responsible for the labeling of one or both particulates then the particulates in Table 2 would have picked up considerably more activity since the concentration of calcium used in these experiments was equivalent to the concentration used in the experiments of Table 1.

Because the preceding experiments (Table 2) were conducted at 2–3°C in non-aerated media, the failure to note any appreciable difference between fresh and prewashed tissue may have been a result of low temperature and the relatively anaerobic system. Therefore, to determine the effect of temperature and aeration on ion uptake by the particulates, fresh and prewashed

Table 3. *Effect of temperature on ion uptake from homogenates prepared from fresh and prewashed tissue. Counts per min. and mg. N.*

Temperature	Mitochondria	Microsomes
Fresh tissue		
2—3°C	116	112
29°C	102	117
Tissue washed 48 hr.		
2—3°C	164	99
29°C	342	111

Uptake of Ca^{45} from the homogenization medium by the mitochondria and microsomes isolated from fresh and prewashed tissue. Equivalent amount of Ca^{45} used in table 1 was added to 100 ml. of homogenization medium.

tissue were homogenized in the presence of Ca^{45} . The homogenate was then separated into 2 equal fractions. One fraction was placed in a temperature control bath at 29°C for 10 min., the other was kept at 2—3°C. Both fractions were similarly aerated. Table III compares the activities of the particulates isolated from fresh and prewashed tissue.

Temperature had no effect upon the ion uptake by the particulates from the fresh tissue; however, the mitochondria from the prewashed tissue exhibited a very striking increase in their ability to bind calcium. At 29°C the mitochondrial activity increased over 100 percent; while the microsomal activity was unaffected. Such a striking increase in the mitochondrial activity in conjunction with their ability to give up calcium in the presence of EDTA is good evidence for the mitochondria being the actual carriers, or the site of carrier molecules, and also indicates that the carriers or their precursors are products of metabolism resulting from metabolic events occurring during the prewash period. The inability of the microsomes to increase their binding capacity may indicate that they function passively in ion uptake as storage sites.

Discussion

Ion exchange during homogenization has heretofore been considered as one of the major obstacles in the interpretation of data concerning ion uptake by the protoplasmic particulates. The data herein presented suggest that very little Ca^{45} is picked up by the particulates during the homogenization procedure, thus indicating that both the mitochondria and microsomes are implicated in the metabolic uptake of ions. This inability of the parti-

culates to bind calcium is not in keeping with Chasson's (1959) observations that both the mitochondria and microsomes of freeze dried material pick up considerable amounts of radiocalcium. It would appear that this inconsistency is largely caused by the methods employed in the preparation and isolation of the particulates. The isolation of the particulates prepared in isotonic sucrose would undoubtedly maintain the mitochondria and microsomes more in their natural state than would their isolation from freeze dried material in ammonium hydroxide-ammonium acetate buffer.

The ability of the mitochondria to release a portion of their bound calcium at a particular concentration of EDTA, and the inability of the microsomes to give up their bound calcium suggests that the mitochondria are the actual carriers or the site of carrier molecules, while the microsomes may function as storage sites.

That the mitochondria are the actual carriers of ions finds further support in the demonstration that mitochondria of prewashed tissue at 29°C exhibit a very striking increase in their ability to bind calcium over mitochondria from fresh tissue. That the microsomes did not exhibit any such increase in their ability to bind calcium indicates that during the prewashing period some metabolic change was initiated within the mitochondria which increased their ability to pick up ions — possibly the production of carrier molecules. These data are in good agreement with the demonstration of Shear and Levitt (1959) that the uptake of calcium by the mitochondria of washed potato slices is greater than the mitochondria of unwashed slices. However, the data herein reported is not in agreement with the observation of Moore *et al.* (1961) wherein they reported that the uptake of calcium by 6-day-old excised barley roots is largely a non-metabolic cell surface adsorption phenomenon. At the present time this discrepancy can not be resolved without further experimentation; however, the difference in experimental material may be part of the explanation.

Summary

1. The data presented indicate that during the homogenization procedure very little calcium is adsorbed by the particulates, thus indicating that both the mitochondria and microsomes are causally concerned in the metabolic uptake of ions.

2. The ability of the mitochondria to release a portion of their bound calcium in the presence of EDTA in conjunction with the fact that prewashing enhances ion uptake by free mitochondria suggests that the mitochondria

are the actual carriers, or the site of carrier molecules, in the metabolic uptake of ions.

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Studies on the Inhibition of Cellulolytic Enzymes

By

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In a previous paper, one of us presented data on the relatively high heat-stability of cellulolytic enzyme preparations from cellulose-decomposing hymenomycetes (Norkrans 1950). Consequently, in an approach to the problem of whether one, two or more extracellular enzymes are involved in the total breakdown of cellulose to glucose, heat-inactivation of cellulolytic enzyme preparations was tried, in order to attain separation of their possible components (Norkrans and Rånby 1956, Norkrans 1957 a, b). It was found that the β -glucoside-splitting activity was eliminated by treatment at 80°C, whereas the cellulose-splitting activity resisted 100°C treatment, although it was strongly reduced.

Conchie and coworkers (1954, 1957) showed that the hydrolysis of β -glycosides can be inhibited by aldonolactones of corresponding configuration. Gluconolactones strongly inhibit β -glucosidases without any action on cellulases, a fact offering new ways for separation of the effect of different components of cellulolytic enzyme preparations (*e.g.* Festenstein 1958).

Material and Methods

The studies were made on enzyme preparations from *Polyporus annosus* Fr. and various strains of *Collybia velutipes* (Curt. ex. Fr.) Quel., the preparations being the same as those used in previous studies (Norkrans 1957 a, b).

By the courtesy of Dr. Elwyn T. Reese, of the Pioneering Research Division Quartermaster Research and Engineering Center, Natick, Mass., a strain of *Trichoderma viride* Pers. ex. Fr. was placed at our disposal, and enzyme preparations were also made from this species.

The fungus was grown at 28°C in liquid culture, 500 ml/2500 ml Fernbach flask, incubated on a reciprocal shaker or — on a larger scale — in a 12-litre vessel containing 8 litres of liquid medium. The vessel was of the so-called "fermenter" type, constructed at the Division of Food Chemistry, The Royal Institute of Technology, Stockholm. The liquid medium had the following composition: cellulose (Munktell no. 3 filter paper, ground in a Turmix blender) 5.0 g, $\text{NH}_4\text{H}_2\text{PO}_4$ 2.0 g, KH_2PO_4 0.6 g, K_2HPO_4 0.4 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, ferric citrate (dissolved in citric acid) 10.0 mg, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 4.4 mg, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 5.0 mg, CaCl_2 100.0 mg, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 1.0 mg, and distilled water 1000 ml. The pH was about 5.8. The medium is a slight modification of that used in previous studies, the modifications being introduced as a consequence of data in a paper by Mandels and Reese (1957) on cellulase production by *Trichoderma viride*. The Fernbach flasks were inoculated with 10 ml of a spore suspension (10^6 – 10^7 spores/ml) from an 8- to 10-day-old malt-agar culture. The fermenter was inoculated with the contents of a Fernbach flask, cultivated for 6 days on the shaker, and inoculated with an amount of spores 10 times that given above. In the fermenter, the liquid medium was aerated by sterile air, 2–3 litres per minute, the air volume being controlled by a flowmeter. At maximum cellulolytic activity in the culture solutions — obtained after an incubation time of about 14 days in the shaking flasks, after 4–5 days in the fermenter — the mycelium was removed. After adjusting the pH of the cell-free filtrate to 4, it was cooled and precipitated in the usual way with 3 volumes of cold acetone. The precipitate was treated in the same way as the acetone precipitates in previous studies.

The protein content was determined with the Folin reagent (Lowry *et al.* 1951). In the *Trichoderma* preparations from the fermenter it amounted to 3100 µg/ml, and in those from the shaking flasks to 2000 µg/ml. The Mi 460 preparation had a protein content of 2000 µg/ml, and the others 850–1000 µg/ml.

Gluconic acid, glucono-1:4-lactone and glucono-1:5-lactone were obtained from Delta Chemical Works, Inc., another glucono-1:5-lactone from British Drug House, arabano-1:4-lactone and galactano-1:4-lactone from Hoffman la Roche.

The β -glucosidase activity was determined with 10^{-3} M *p*-nitrophenyl- β -glucoside as substrate, entirely in accordance with the details given in previous papers, in which a full account is also given of the turbidimetric method for determination of cellulase activity. In the present investigation the turbidimetric measurements were, however, made in tubes in a Beckman B spectrophotometer at 610 m μ , using a cellulose sol containing 10 mg/ml of cellulose. Reducing values were determined and calculated as glucose, by means of the dinitrosalicylic acid method of Sumner and Sommers (1944), and the amount of glucose formed by the method of Hilf *et al.* (1959).

Results and Discussion

Inhibition of β -glucosidase activity. The effect of gluconic acid and of some aldonolactones on the hydrolysis of 10^{-3} M *p*-nitrophenyl- β -glucoside ("niphel-gluc") was determined. Gluconic acid, as well as glucono-1:4-lactone and glucono-1:5-lactone, were tested in concentrations ranging from 10^{-7} to 10^{-2} M, whereas galactano-1:4-lactone and arabano-1:4-lactone were used in concentrations of 10^{-2} or 10^{-3} M. The tests were usually run up to 60 min-

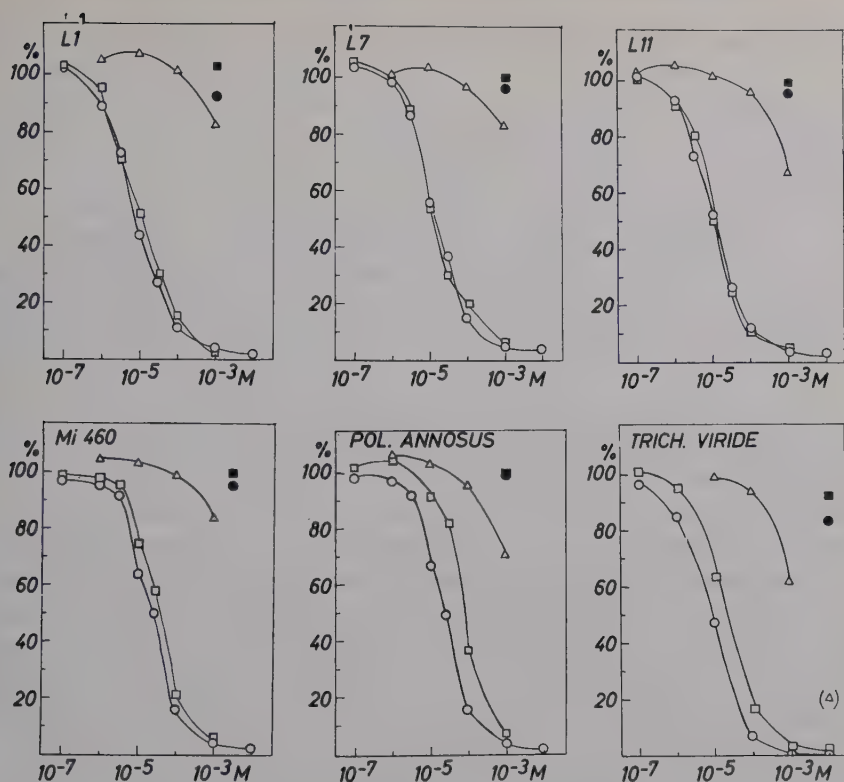


Figure 1. Relative activity of *p*-nitrophenyl- β -glucosidase, plotted as percentage of the control, from some strains of *Collybia velutipes*, *Polyporus annosus*, and *Trichoderma viride* at various concentrations of gluconic acid and some aldonolactones,

- gluconic acid
- glucono-1:5-lactone
- △—△ glucono-1:4-lactone
- arabano-1:4-lactone
- galactano-1:4-lactone

utes, measurements being made at intervals during this period. In the niphegluc test, there is generally a direct proportionality up to at least 60 per cent total cleavage of niphegluc (Norkrans 1957 a). The data were therefore selected from that reaction time where the control value amounted to maximally about 60 per cent. In Figure 1, the hydrolysis values from series with addition of the various test substances are given as a percentage of the corresponding control value. As can be seen from the Figure, both gluconic acid and glucono-1:5-lactone inhibited all enzyme preparations tested to approximately the same extent. At a concentration of 10^{-5} M, the activity was re-

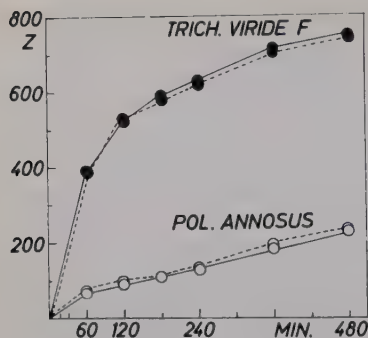


Figure 2. Effect of 10^{-3} M gluconic acid on the cellulase activity of *Trichoderma viride* and *Polyporus annosus*, measured turbidimetrically. Series without gluconic acid plotted as solid lines, with gluconic acid as broken lines.

duced to about 50 per cent, the corresponding value at a concentration of 10^{-3} M being 0, or a mere 5–7 per cent.

Glucono-1:4-lactone, on the other hand, had no inhibitory effect at a concentration of 10^{-5} M, and at 10^{-3} M produced an inhibition of only 20 per cent in all *Collybia* preparations with the exception of L 11. At the latter concentration, L 11 and *Polyporus annosus* lost 30 per cent of their activity, and *Trichoderma viride* 40 per cent. At higher concentrations, glucono-1:4-lactone produced slight precipitation, which interfered with the spectrophotometer readings.

Reese *et al.* (1959), in a study of fungal β -glucosidases, pointed out that the glucono-1:4-lactone is somewhat less inhibitory than the glucono-1:5-lactone. The rumen β -glucosidases tested by Conchie (1954) and others were, however, found to behave in the opposite way, since they were strongly inhibited by both lactones, whereas gluconic acid had no appreciable inhibitory effect. The inhibition of β -glucosidase activity by the gluconolactones has been attributed to their similarity to glucose, a cleavage product of the enzymic hydrolysis. Lactones with another configuration are less powerful inhibitors, here reflected in the slight inhibitory effect of the arabano and galactano-lactones. The glucono-1:4-lactone corresponding to the furanose form of glucose was also less effective than the glucono-1:5-lactone, corresponding to the pyranose form.

Inhibition of cellulase activity in turbidimetric test. The effect of glucono-1:5-lactone and gluconic acid in various concentrations on the cellulase activity was determined turbidimetrically. At a concentration of 10^{-2} M, the *Collybia* preparations were slightly inhibited, showing a maximum reduction in activity of 18 per cent (L7 and Mi 460), and about the same inhibition at a concentration of 10^{-3} M. *Polyporus annosus* was inhibited by 7 per cent at a concentration of 10^{-2} M; at 10^{-3} M, however, the inhibition was

completely abolished, as can be seen in Figure 2. This also applied to the *Trichoderma viride* preparation.

The turbidimetric test unquestionably reflects the splitting of long chains, a cellulase activity which is evidently *not* inhibited by gluconic acid in a concentration of 10^{-3} M, whereas the β -niphogluc-splitting activity was completely inhibited. This β -glucosidase active against an aryl- β -glucoside does not, however, necessarily represent all β -glucosidase activity in the enzyme preparations, nor does it necessarily represent the whole effect of the cellobiase (*cf.* Youatt *et al.* 1959) *i.e.*, the β -glucosidase which, in studies concerning total cellulose breakdown, may be of main interest.

Differentiation of cellobiase and cellulase activity by means of gluconic acid in a concentration of 10^{-3} M. In the following experiments, the cellulolytic activity was measured against cellulose and cellobiose. Cellobiose was used in 0.5 per cent solution. The hydrolysis of cellobiose was denoted as cellobiase activity. The activity was measured in terms of glucose formed. Cellulase tests were run with cellulose sol as substrate at two concentrations, 0.5 and 0.1 per cent, respectively. The enzyme activity is expressed in terms of reducing substances, calculated as glucose, as well as in terms only of the glucose formed.

Polyporus annosus. In series without gluconic acid, about 15 per cent of the total amount of cellobiose had been hydrolyzed to glucose already after 60 minutes, and 20 per cent after 4 hours. On addition of gluconic acid, no cellobiase activity could be demonstrated in the *Polyporus* preparation after an incubation time of up to 2 hours. After 2 hours, a certain amount glucose was sometimes formed (Figure 3, *a* and *b*).

The cellobiase activity could evidently be inhibited by gluconic acid. When substituting gluconic acid for the less stable gluconolactones — in our experiments, the glucono-1:5-lactone could have been used — the incubation periods need not be restricted to the maximal 30–60 minutes recommended by Festenstein (1958) for inhibition studies with gluconolactones.

Addition of gluconic acid to the series with 0.1 per cent cellulose brought about an inhibition of about 50 per cent in the formation of reducing substances, after the first 30 minutes had elapsed. This is presumably to be ascribed to inhibition of the cellobiase activity.

During the first 30 minutes, however, the difference between the amount of reducing substances formed in the series with and without gluconic acid was only about 20 per cent. This implies that any marked cellobiase-inhibiting effect by gluconic acid could not be observed until the cellulase had released a certain quantity of cellobiose molecules.

As already mentioned, the preparation in question does, in fact, have high cellobiase activity and, consequently, a high production of glucose in the

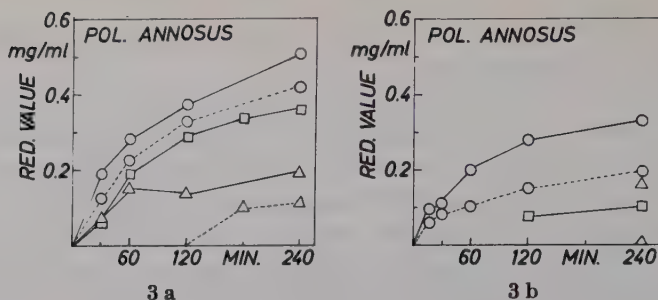


Figure 3 a. Effect of 10^{-3} M gluconic acid on the production of reducing substances by *Polyporus annosus* preparation from 0.5 % cellulose solution (○—○ measured as reducing substances, calculated as glucose, □—□ measured as glucose) and from 0.5 % cellobiose solution (△—△ measured as glucose). All values in mg/ml. Series without gluconic acid plotted as solid lines, with gluconic acid as broken lines.

Figure 3 b. Effect of 10^{-3} M gluconic acid on the production of reducing substances by *Polyporus annosus* preparation from 0.1 % cellulose solution, (○—○ measured as reducing substances, calculated as glucose, □—□ measured as glucose) and from 0.5 % cellobiose solution (△—△ measured as glucose). All values in mg/ml. Series without gluconic acid plotted as solid lines, with gluconic acid as broken lines.

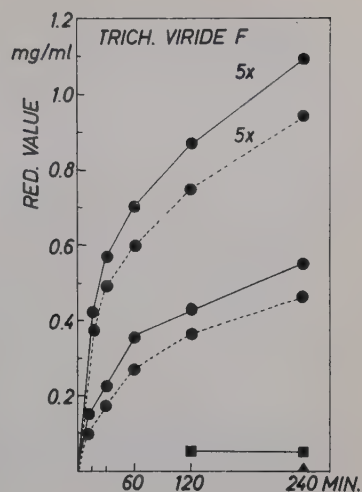
cellulase tests. It can be inferred from Figure 3 a that the glucose amounted to about 60 per cent or more of the total quantity of reducing substances after all test periods (56, 70, 74, and 65 per cent after an incubation time of 30, 60, 120, and 240 minutes, respectively).

In the series of 0.5 per cent cellulose, on the other hand, the inhibition measured in reducing values was not more than about 20 per cent (Figure 3, a). It was thus of about the same size as the inhibition in the 0.1 per cent series during the first 30 minutes.

Assuming that the cellulase activity has a higher affinity to a long chain of anhydroglucose units (*cf.* Whitaker 1959), only small amounts of cellobiose will be formed as long as cellulose substrate is available in excess. In the 0.5 per cent series this applied during the whole test period, but in the 0.1 per cent series only during the first 30 minutes.

Trichoderma viride. The *Trichoderma viride* F preparation, obtained by culture in the fermenter, had an entirely different course of cellulose breakdown (Figure 4) to that of *Polyporus annosus*. Its cellobiase activity was extremely weak. Thus, in the cellobiase test, only traces of glucose were formed during a 4-hour incubation period. After 7 hours, 0.2 mg/ml had been formed in the uninhibited series, an activity which was completely inhibited by gluconic acid, since no glucose could be detected in the series with

Figure 4. Effect of 10^{-3} M gluconic acid on the production of reducing substances by *Trichoderma viride* preparation F. from cellulose solutions, 0.5 (5x) and 0.1 %, respectively (●—● measured as reducing substances, calculated as glucose, ■—■ measured as glucose) and from 0.5 % cellobiose solution (▲—▲ measured as glucose). All values in mg/ml. Series without gluconic acid plotted as solid lines, with gluconic acid as broken lines.



gluconic acid. The glucose formation from cellulose as substrate was also exceedingly weak, although the overall cellulolytic reaction rate was much more rapid than in *Polyporus annosus*. After an incubation time of 120 minutes, for instance, 0.07 mg/ml of glucose and 0.86 mg/ml of reducing substances were formed by the *Trichoderma* preparation, whereas the corresponding values for the *Polyporus* preparation were 0.28 and 0.38 mg/ml, respectively. Even though no cellobiase activity could be observed during the first 4 hours, the formation of reducing substances was inhibited at a practically constant rate at both cellulose concentrations. Consequently, in this case, the inhibition of about 15 per cent could be ascribed to an inhibition of cellulase. Since an endwise attack on cellulose — in addition to the random splitting activity — has been found in *Trichoderma viride* preparations (Gilligan and Reese 1954), the cellulase inhibition can tentatively be attributed to an inhibition of this action. It seems reasonable to assume this type of cellulase activity to be more sensitive to the competitively inhibitory effect of the glucose molecule than the cellulase with a preferentially random splitting activity.

Figure 5 shows the data from another *Trichoderma viride* preparation, i.e., from shaking cultures, as well as some data from a fraction of it obtained by zone-electrophoretic separation.

The unfractionated preparation had weak cellobiase activity; glucose was detected only after an incubation period of 270 minutes. In the cellulase test, the formation of reducing substances was inhibited by 30–40 per cent by addition of gluconic acid, and the glucose-forming ability was much more pronounced in this shaking culture preparation (117 S), than in the fer-

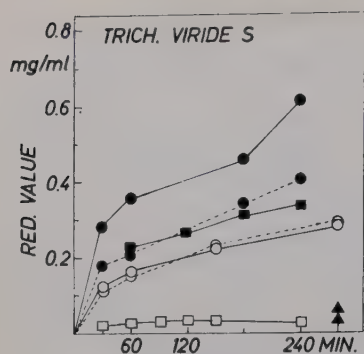


Figure 5. Effect of 10^{-3} M gluconic acid on the production of reducing substances by *Trichoderma viride* preparation 117 S from 0.5 % cellulose solution (●—● measured as reducing substances, calculated as glucose, ■—■ measured as glucose) and from 0.5 % cellobiose solution (▲—▲ measured as glucose). All values in mg/ml. Series without gluconic acid plotted as solid lines, with gluconic acid as broken lines. Unfilled circles and squares represent the corresponding series for the fractionated *Trichoderma viride* 117 S.

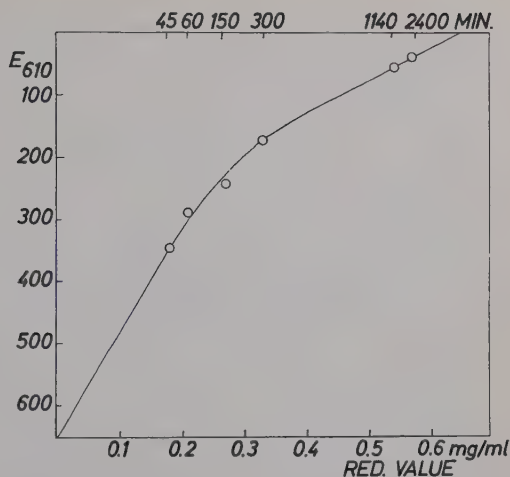
menter preparation. Another shaking culture preparation behaved in the same way, yielding 0.1, 0.12, 0.18, 0.19, 0.23, and 0.28 mg/ml of glucose after incubation periods of 30, 60, 90, 120, 180, and 240 minutes, respectively. The physical cultural conditions under which different preparations are obtained evidently play some role not only in the degree of cellulolytic activity, but also in its nature, a fact to which Thomas (1956) has drawn attention. (Some studies made by us on the influence of cultural conditions on cellulase formation will be published elsewhere).

We sometimes found a decrease in the amount of glucose obtained from cellobiose with time, which could be explained as a result of transglucosidase activity (Whitaker 1959). Some paperchromatographic studies made concurrently with the present investigation showed the formation of polysaccharides. The presence of cellotriose (for which we had a pure reference substance) has been definitely demonstrated.

Figure 5 also shows the activity of a fraction of the enzyme preparation 117 S. Fractionation was performed by Dr J. Porath of the Institute of Biochemistry, Uppsala, as follows. The acetoneprecipitated 117 S was desalted by Sephadex G 25, freeze-dried, and run in zone electrophoresis in a Tris buffer, pH 7.8, at an ionic strength of 0.05. The top material from the main peak with cellulase activity was used. Gluconic acid did not inhibit the activity measured in terms of reducing substances. Glucose was formed only in minute amounts from cellulose, and not at all from cellobiose. This seems to denote that all β -glucosidase activity and probable endwise-acting cellulase activity had been removed.

Finally, Figure 6 shows the correlation between the cellulase activity of *Trichoderma viride* 117 S, measured as the reducing substances formed and with the turbidimetric method. During the first hour of incubation the turbidimetric method reflects only the effect on the long chains of, anhydroglucose units, whereas the other test includes all activities on β -glucosidic

Figure 6. Correlation between the cellulase activity of *Trichoderma viride* 117 S in 0.1 % cellulose solution, expressed in terms of reducing substances (mg/ml), and E_{610} values from the turbidimetric test after various test periods (min).



linkages. A tangential continuation of the curve, constructed through the point at 60 minutes indicates that about 60 per cent of the reducing substances (about 0.4 mg of totally 0.65 mg/ml) may be formed by the random splitting cellulase activity. The remaining 40 per cent could be ascribed to β -glucosidase activity and an endwise cellulase attack, well corresponding to the abovementioned values for inhibition by the gluconic acid with the same enzymic preparation.

This investigation has been supported by a grant from the Swedish Natural Science Research Council.

Summary

1. It is found that gluconic acid and glucono-1:5-lactone in a concentration of 10^{-3} M completely inhibit *p*-nitrophenyl- β -glucosidase, and produce 50 per cent inhibition at a concentration of 10^{-5} M.

2. Cellobiase activity is inhibited by gluconic acid at the same concentration, the cellobiase activity of *Polyporus annosus* being much stronger than that of *Trichoderma viride*.

3. Cellulase activity on long anhydroglucose chains, measured in turbidimetric tests, is not inhibited by addition of gluconic acid or glucono-1:5-lactone at a concentration of 10^{-3} M.

4. The cellulase activity of *Trichoderma viride* (F), measured in terms of reducing substances, is inhibited at a constant rate to about 15 per cent, tentatively explained as an inhibition of the endwise cellulase attack.

5. It is suggested that differences in the physical conditions of culture influence not only the degree of activity, but also the nature of the enzymes.

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The Inhibitor β Complex from Resting Potato Tubers as an Inhibitor of α -Amylase

By

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Introduction

Sukhorukov, Kling and Ovčarov (1938) have demonstrated in tubers and leaves of potato the presence of a water-soluble amylase inhibitor, which occurs in larger amounts in ungerminated potato than in germinated. If the tubers are infected with *Phytophthora infestans* the inhibitor disappears rapidly. Already 24 hours after the infection a marked decrease in the inhibitor content can be shown.

Bowman (1945) found in "navy beans" a water-soluble, thermolabile amylase inhibitor, which can be precipitated from an aqueous solution by alcohol. In wheat endosperm Kneen and Sandstedt (1946) discovered a water-soluble amylase inhibitor, which is resistant to boiling at 100°C but is destroyed by autoclaving. It is, furthermore, insoluble in, i.e., concentrated ammonium sulphate solution, ether and 90 per cent alcohol and cannot pass through a dialyzing membrane. Militzer, Ikeda and Kneen (1946 a and b) have studied the properties of this inhibitor and consider it to be a protein.

In the husks of *Avena sativa* Elliott and Leopold (1953) found a water-soluble amylase inhibitor, affecting both α - and β -amylase. It is not dialyzable and cannot be precipitated by saturated ammonium sulphate solution.

In peelings of resting potato there occur acid growth-inhibiting substances, which disappear when the potato leaves the rest period naturally or through treatment with a rest-interrupting agent (for literature see Hemberg, 1958 and in press). It is believed that these acid growth inhibitors are composed of the so-called inhibitor β complex (Benett-Clark and Kefford 1953). Marinos

and Hemberg (1960) have shown that inhibitor β from peelings of resting potato not only inhibits the growth of segments of *Avena* coleoptiles but also stimulates the oxygen uptake of these segments as well as of discs of tissue from non-resting potato tubers. Moreover, inhibitor β represses the inorganic phosphate uptake of these potato discs. These investigators assume that inhibitor β "may bring about its growth-inhibiting effect by uncoupling phosphorylation from the electron-transferring systems and by this means deprive the tissue of energy (ATP) necessary for the performance of the synthetic reactions associated with growth".

The present investigation is intended to elucidate whether inhibitor β in addition to the effect on respiration and growth demonstrated by Marinos and Hemberg also influences the activity of some carbohydrolytic enzyme systems.

Material and Methods

As the source of inhibitor β peelings of resting potato of the variety Majestic were employed. The inhibitor was extracted by ether and purified by fractionation and paper chromatography according to methods previously described (Hemberg 1958, Marinos and Hemberg 1960). Since transcinamic and salicylic acids have been considered by certain investigators (Köves 1957, Varga 1957, Köves and Varga 1958) to constitute some of the components in the inhibitor β complex, the effect of these substances has also been investigated.

The enzymes studied have been primarily α - and β -amylase, which have been obtained from the firm Theodor Schuchardt, Munich. Some experiments have also been performed with invertase and cellulase. The former has been placed at our disposal by Professor K. Myrbäck, Stockholm University, and the latter by Dr. Birgitta Norkrans, Royal Institute of Pharmacy, Stockholm.

The amylase activity has been determined according to Willstätter and Schudel (1918) by the addition of iodine solution and titration of the unconsumed iodine with sodium thiosulphate, whereafter the amount of glucose formed can be calculated.

The invertase activity has been determined according to Fischer and Kohtès (1951) by adding to the samples a solution containing dinitrosalicylic acid, sodium hydroxide and potassium-sodium tartrate. On boiling the samples a red color is obtained in the presence of reducing sugar. The intensity of the color is increased with increasing amounts of reducing sugar and can be determined spectrophotometrically at 5,500 Å.

The cellulase activity has been determined according to Norkrans (1950) by spectrophotometric examination at 6,100 Å of how the extinction of a cellulose sol is decreased by the activity of the enzyme. The more the cellulose is decomposed the clearer the solution and the lower its extinction.

Results and Discussion

In one first experimental series the effect of inhibitor β , transcinamic acid and salicylic acid on a mixture of equal amounts of α - and β -amylase was

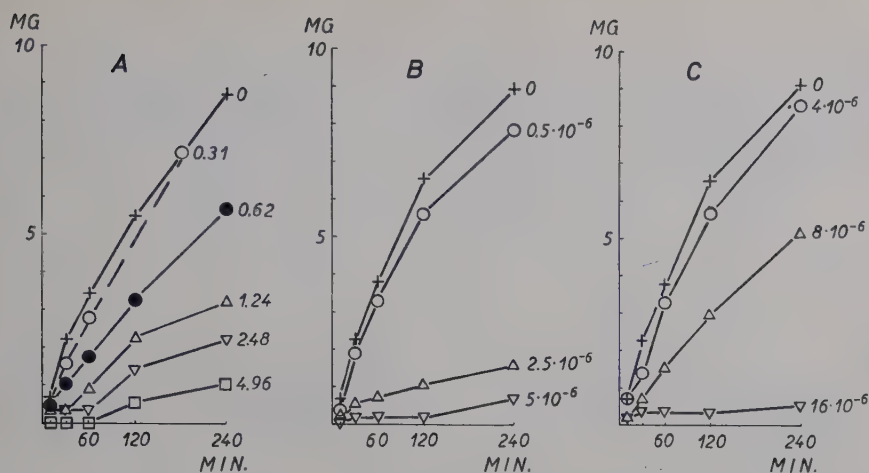


Figure 1. The effect of inhibitor β (A), transcinnamic acid (B) and salicylic acid (C) on a mixture of α - and β -amylase. The figures adjacent to the different curves give for A the amount of inhibitor β , expressed as the number of grams of potato peel used per ml. of test solution; for B and C the molarity of the experimental solutions of the synthetic inhibitors employed. The experimental volume was 30 ml., 20 ml. of which were composed of a 1 per cent starch solution in $\frac{1}{15}$ M phosphate buffer, pH 5.0. The enzyme concentration was 0.008 mg. of each enzyme per ml. experimental solution. Abscissa: Time in minutes after start of the experiments. Ordinate: Mg. glucose formed.

studied. The results, which are given in Figure 1, show that all three substances clearly inhibit the amylase activity.

In continued experiments the effect of the three substances on only α -amylase or only β -amylase was investigated. Table 1 shows the results of these experiments, some of which were performed at 20°C and others at 30°C. From the table it is evident that all three of the investigated substances strongly inhibit the activity of α -amylase. β -amylase, on the other hand, is inhibited not at all by salicylic acid, possibly somewhat by inhibitor β and somewhat by transcinnamic acid.

Elliott and Leopold (1953) assumed that their inhibitor was a polypeptide, i.e., because it was not dialyzable through a cellophane membrane. Inhibitor β , on the other hand, is not considered to be a polypeptide. For investigation of the dialyzing properties of the latter the following experiments were carried out. Inhibitor β from potato peel, purified by paper chromatography in isopropanol-ammonia-water, was dissolved in distilled water and divided into four portions. Two of these were transferred to dialyzing tubes and dialyzed under stirring in frequently changed distilled water for 18 and 42 hours respectively. The other portions served as controls and were allowed to stand

Table 1. *Inhibition of the activity of α - and β -amylase under the influence of inhibitor β , transcinamic and salicylic acids expressed in per cent of the activity in the control experiments without addition of the inhibitory substances. Experimental volume 30 ml., thereof 20 ml. 1 per cent starch solution. The starch in the α -amylase experiments was dissolved in 0.02 M sodium acetate buffer, pH 4.7; in the β -amylase experiments dissolved in $1/15$ M phosphate buffer, pH 5.9. The concentration of the enzymes was 0.0167 mg. per ml. experimental solution. In the experiments at 20°C the glucose content was determined after 60 minutes, in those at 30°C after 30 minutes.*

Exp. temp. °C.	Inhibitor β			Transcinamic acid			Salicylic acid		
	"β" g./ml.	% inhibition of activity of		Molar conc.	% inhibition of activity of		Molar conc.	% inhibition of activity of	
		α -amy- lase	β -amy- lase		α -amy- lase	β -amy- lase		α -amy- lase	β -amy- lase
20	3.1	27	0	$2.5 \cdot 10^{-5}$	45	3	$1.2 \cdot 10^{-5}$	25	0
	7.5	40	0	$5 \cdot 10^{-5}$	66	8	$3.1 \cdot 10^{-5}$	68	0
30	7.5	76	4	$5 \cdot 10^{-5}$	54	11			
	7.5		4						

in test tubes for correspondingly long periods. After completed dialysis, both the dialyzed portion and the corresponding control were acidified and thereafter shaken with ether which then was evaporated to a fixed volume. Part of each ether solution was chromatographed, and that part of the chromatogram where inhibitor β should occur was tested with the "straight growth" test according to Hemberg (1958). The test plants employed were *Avena coleoptiles* of the variety Brighton. The rest of the ether solutions were evaporated to dryness and shaken with distilled water, whereafter the effect of the aqueous solutions on α -amylase was determined. It was shown, see Figure 2 and Table 2, that the dialyzed inhibitor β extract did not have any inhibitory effect in the "straight growth" test or on the activity of α -amylase. In Figure 2 and Table 2 are also presented experiments showing that inhibitor β is not destroyed by heating up to 100 or 120°C but that the substance despite this treatment has an inhibiting effect on the growth of the coleoptile segments as well as on the activity of α -amylase.

Since inhibitor β from potato peel is dialyzable and affects only α -amylase and not β -amylase, it cannot be identical to the amylase inhibitor demonstrated by Elliott and Leopold. On the other hand, it is possible that the amylase inhibitor discovered by Sukhorukov, Kling and Ovčarov (1938) in potato might be identical to inhibitor β . Inhibitor β disappears, as previously mentioned, in connection with the natural breaking of the rest period. Also on treatment of resting potato with rest-breaking agents such as ethylene chlor-

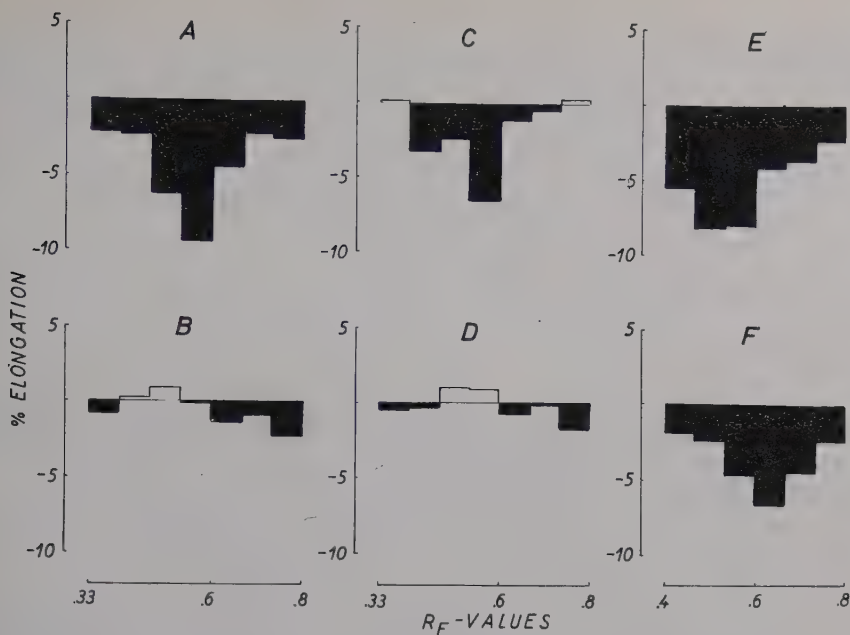


Figure 2. The effect of aqueous extracts of inhibitor β treated in different ways on the "straight growth" of oat coleoptile segments. After the treatment the extracts were chromatographed in isopropanol-ammonia-water, and the parts of the chromatograms where the inhibitor β complex normally occurs were tested with the "straight growth" test. In the chromatograms A-D inhibitor from 9.8 g. peel, in E-F inhibitor from 9.7 g. A and C: the extracts kept in aqueous solution at room temperature for 18 and 42 hours respectively. B and D: the extracts dialyzed for 18 and 42 hours respectively. E: the extracts boiled in a water bath for 40 minutes. F: the extract autoclaved at 120°C for 40 minutes. Abscissa: Position of the paper segment on the chromatogram in R_f units. Ordinate: Growth of oat coleoptile segments in per cent of growth of the control segments.

Table 2. The effect of extracts of inhibitor β treated in different ways on the activity of α -amylase expressed in per cent of the activity in the control experiments without addition of inhibitor. Experimental volume 6 ml., thereof 4 ml. 1 per cent starch solution in 0.02 M sodium acetate buffer, pH 4.7. Enzyme concentration 0.02 mg. per ml. Inhibitor β from 13.4 g. potato peel per ml. Experimental temperature 30°C. The glucose content determined 30 minutes after the beginning of the experiment.

Treatment of inhibitor extract	Per cent inhibition of enzyme activity
Dialyzed for 18 hours	0
Not dialyzed, kept in aqueous solution at room temperature for 18 hours	79
Dialyzed for 42 hours	0
Not dialyzed, kept in aqueous solution at room temperature for 42 hours	69
Boiled in water bath for 40 minutes	81
Autoclaved at 120°C for 40 minutes	90

Table 3. *Investigation of the activity of invertase with and without addition of inhibitor β . Experimental volume 10 ml., thereof 5 ml. 5 per cent sucrose solution in $1/15$ M phosphate buffer, pH 5.0, 2.5 ml. diluted invertase solution and 2.5 ml. inhibitor β in aqueous solution. Inhibitor β from 5 g. potato peel per ml. experimental solution.*

Time in min. after start	Extinction		
	Samples without invertase	Samples with invertase and	
		Without β	With β
0	0.10	—	—
3	—	0.85	0.86
6	—	1.3	1.2
9	—	1.4	1.4
12	—	1.6	1.6

Table 4. *Investigation of the activity of cellulase with and without addition of inhibitor β . Experimental volume 6 ml., thereof 1 ml. cellulase solution and 1 ml. cellulose sol in phosphate buffer, pH 5.0.*

No. of hrs. after start	Difference between the extinction at the beginning of the experiment and at the different times of measurement			
	Control	Inhibitor from		
		4.2 g. peel/ml.	8.3 g. peel/ml.	16.7 g. peel/ml.
1	0.230	0.232	0.230	0.214
2	0.274	0.287	0.260	0.279
4	0.355	0.367	0.347	0.359
22	0.555	0.527	0.533	0.554

hydrin, glutathione or gibberellic acid the inhibitory substances disappear and the rest is broken. (Hemberg 1949, 1950, Boo 1961). Dostal (1942) has shown that potato infected by *Phytophthora* germinates immediately after harvesting. Thus it does not have a rest period. One can therefore assume that such a potato has relatively little inhibitor β . The absence of this inhibitor in *Phytophthora*-infected potato might be able to explain why the aqueous extract of such a potato does not have an inhibitory effect on amylase, whereas the extract of uninfected potato does.

In Tables 3 and 4 experiments are presented showing that inhibitor β affects neither the activity of invertase nor that of cellulase.

Summary

Inhibitor β from peelings of resting potato, purified by paper chromatography (isopropanol-ammonia-water) represses the activity of α -amylase but very insignificantly the activity of β -amylase. Also transcinamic and salicylic acids exercise an inhibitory action on α -amylase. The first-mentioned inhibits also, if only to a slight extent, the activity of β -amylase.

If an aqueous extract of inhibitor β is dialyzed, the ability of the extract to repress both the "straight growth" of coleoptile segments and the activity of α -amylase disappears. The activity of the inhibitor is not affected, on the other hand, by heating in an aqueous solution to 120°C for 40 minutes.

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Chlorophyll Concentration and Rate of Photosynthesis in *Chlorella vulgaris*

By

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1. Introduction

The light absorbed by several pigments provides the energy in photosynthesis. However, present knowledge (cf. *e.g.* Rabinowitch 1951) indicates that with the exception of photo-autotrophic bacteria only chlorophyll-*a* is able directly to transfer light energy into chemical energy. The light energy absorbed by the other active pigments is first transferred to associated chlorophyll *a* molecules.

The dependence of the rate of photosynthesis on the concentration of chlorophyll — and the other active pigments — has been studied relatively little. Until recently this dependency was investigated practically only at light saturation. Gabrielsen (1948), however, stressed the importance of studying the influence of chlorophyll concentration at low light intensities, where the rate of the overall photosynthetic process is limited by the rate of the photochemical part of the process. Chlorophyll concentration is a factor of direct influence on photosynthetic rate only at low light intensities. This fact was pointed out already by Harder (1923).

At light saturation, where the overall rate of photosynthesis is determined by the rates of enzymatic processes, it is by no means a matter of course to find any relation between the concentrations of the pigments active in photosynthesis and the rate of photosynthesis. If a relation is found nevertheless, this must be due to the existence of a correlation, *e.g.*, between the concentration of the pigments and the concentration of the photosynthesis enzymes. In their classical studies, Willstätter and Stoll (1918) showed that such a

correlation is not always found. Leaves containing very small amounts of chlorophyll — leaves of aurea varieties — may have a high rate of photosynthesis at light saturation.

In leaves of higher plants, where chlorophylls *a* and *b* are probably the absolutely dominating photosynthesis pigments, Gabrielsen (1948) showed that even at low light intensities the rate of photosynthesis per unit area is independent of the concentration of chlorophyll, if the latter is higher than about 6 mg (*a* + *b*) per dm², as found in most leaves. Only if the concentration is below 1 mg per dm² — i.e., in extremely chlorophyll poor leaves — is a direct proportionality found between the rate of photosynthesis and the concentration of chlorophyll. According to Gabrielsen, at such low chlorophyll concentrations 0.28 mg *C* is assimilated per mg chlorophyll *a* + *b* per hour, if the light intensity (0.4–0.7 μ) is 1 cal. per m² per second (incandescent light). In ordinary green leaves (about 5–7 mg chlorophyll *a* + *b* per dm²) 0.07–0.09 mg *C* is assimilated per mg chlorophyll *a* + *b* per hour at the same light intensity.

It is thus evident that chlorophyll is normally present in excess in leaves of higher plants. In dilute suspensions of plankton algae this can hardly be the case. In plankton algae therefore at a low light intensity we have to expect a photosynthetic rate measured per unit of chlorophyll of the same order of magnitude as in the very chlorophyll poor leaves of the so-called aurea varieties. According to Gabrielsen (1960) light absorption is relatively high in leaves completely devoid of photosynthesis pigments — about 27 per cent (0.4–0.7 μ). In plankton algae light absorption not due to the pigments active in photosynthesis is very likely of somewhat less importance than in the leaves of the aurea varieties. Therefore per unit of chlorophyll we may expect somewhat higher rates of photosynthesis at low light intensities than in the “aurea” leaves.

No adequate measurements seem to have been made in plankton algae with the exception of the rather special case of a blue green alga (cf. p. 873) correlating the rate of photosynthesis at a low light intensity with the concentration of chlorophyll. Emerson (1929) who used *Chlorella* for such measurements — but used arbitrary units both for light intensity and rate of photosynthesis — believed that the rate of photosynthesis was independent of the concentration of chlorophyll at low light intensities. Using Emerson's own measurements Gabrielsen (1960) was able to show the incorrectness of Emerson's statement.

Steemann Nielsen and Aabye Jensen (1957) attempted to obtain for plankton algae in general an estimate of the approximate rate of photosynthesis per unit of chlorophyll at low light intensity, but were forced to combine the results of investigations made by two different workers. The rate of

photosynthesis in the green alga *Chlorella pyrenoidosa* was taken from Winokur (1948) and the concentration of chlorophyll from Kok (1953). Chlorophyll *a* and *b* are likely the absolutely dominating photosynthesis pigments in the green alga *Chlorella*. As a first approximation such a procedure was considered to be adequate. It was calculated that 0.36 mg *C* is assimilated per mg chlorophyll *a+b* per hour at 1000 lux (incandescent light) computed per one cal. per m² per second (0.4–0.7 μ), 0.46 mg *C* should thus be assimilated per mg chlorophyll *a+b* per hour in *Chlorella pyrenoidosa*.

Steemann Nielsen and Hansen (1959 b) retained the rate 0.36 mg *C* per mg chlorophyll *a+b* per hour at 1000 lux for ordinary surface algae. For typical shade plankton they suggested instead a ratio 0.29 (cf. below).

According to these estimates of the photosynthetic rates at low light intensities *Chlorella* should photosynthesize per unit of chlorophyll about 40 to 60 per cent better than the leaves of the aurea varieties of the higher plants. Since the estimates were first approximations only, special experiments have now been made. The rate of photosynthesis and the concentration of chlorophyll *a+b* have been measured simultaneously in *Chlorella vulgaris*. At the same time the influence of chlorophyll concentration per cell volume in *Chlorella* was investigated. As shown by Duysen and Huiskamp (according to Rabinowitch 1956) the light absorption in a single cell of *Chlorella* is relatively high. Some influence of the pigment concentration in the cell on the rate of photosynthesis at a low light intensity per unit of chlorophyll must thus be expected.

2. Experiments

Chlorella vulgaris (Cambridge Culture Collection 211/11 h) was grown in continuous incandescent light — either at 3,000 or 30,000 lux — at 21°C. Special containers of about 150 ml capacity (Jørgensen and Steemann Nielsen 1961) aerated with atmospheric air were used. The containers were placed in a water bath. The density of the suspensions which were stirred by rapid bubbling of atmospheric air was in all cases so low that at the most 30 per cent of the area of the cross section of the containers would have been covered with algae if these had been arranged in a monolayer. Österlind *B* medium (Österlind 1949) with phosphate buffer at pH 6.5 was employed. The algae increased by a factor 2.0 during 24 hours at 3,000 lux and by a factor 5.4 at 30,000 lux.

The carbon-14 technique was used for measuring the rate of photosynthesis. The special apparatus with a rotating wheel carrying duplicates of experimental bottles at 7 different light intensities — Schott neutral glass — as described by Steemann

Nielsen and Hansen (1961) was employed. Eight different light intensities may be used, if duplicates only are found at six of these light intensities. This technique has the advantage that high and low rates of photosynthesis may be measured with the same percentage precision. The density of the suspension of algae used in the photosynthesis experiments was extremely low, only 10 per cent of that used during culture (same medium with the addition of 3 mM KHCO_3 per liter and C-14 ampoules). As the light source including thirteen 100 Watt bulbs covers an area of 35 cm \times 35 cm and the diameter of the experimental bottles is only about 2 cm, all algae in a bottle are illuminated uniformly. The duration of a single experiment was 30 minutes. The temperature was 21°C. The rates were corrected to real photosynthesis (cf. Steemann Nielsen and Hansen 1959).

The concentration of chlorophyll *a* and *b* was determined individually in the two component mixtures from measurements of the optical density at the red peak of each component. The data of Richards 1952 for specific absorption coefficients in 90 per cent aqueous acetone, and the optical densities at 663 and 645 m μ were used to calculate concentrations of both chlorophylls *a* and *b*. The extraction of chlorophyll by 90 % acetone without disintegrating the cells was somewhat difficult, although the algae were boiled for one minute in the acetone. As the extraction by methanol was always found to be complete, aliquots of algae extracted with methanol were used for controlling and correcting the measurements obtained by means of the samples extracted with 90 % acetone. Chlorophyll is expressed as mg (*a* + *b*) per mm³ of wet volume of the algae. Wet volumes were obtained by centrifugation according to Winokur (1948).

The wet volumes per 10⁸ cells were 17.5 and 25.4 mm³ for the cultures grown at 30,000 and 3,000 lux, respectively. The average diameter of the cells was 5.6 μ for those grown at 30,000 lux and 6.5 μ for those grown at 3,000 lux. About 300 cells were measured in each culture.

2.4 μ g chlorophyll *a* + *b* was found per mm³ algae in the culture grown at 30,000 lux and 5.7 μ g in that grown at 3,000 lux. Using a conversion factor of 4 for volume: dry weight (cf. Winokur 1948, table 4) the percentage of chlorophyll *a* + *b* per dry weight is 0.93 in the 30,000 lux culture, and 2.2 in the 3,000 lux culture.

According to the information given above, the cells of the present strain of *Chlorella vulgaris* are relatively large. Strains of *Chlorella vulgaris* with higher maximum concentrations of chlorophyll are known, but the present strain can by no means be characterized as being poor in chlorophyll. As might be expected, the cells grown at the low light intensity are considerably richer in chlorophyll than those grown at the high light intensity. In both cultures the concentration of chlorophyll *b* was about one third of that of chlorophyll *a*.

Figure 1 presents the rate of photosynthesis (mg C per mg chlorophyll *a* + *b* per hour) as a function of light intensity for *Chlorella vulgaris* grown at 30,000 and 3,000 lux, respectively. Measured in that way, the rate at light

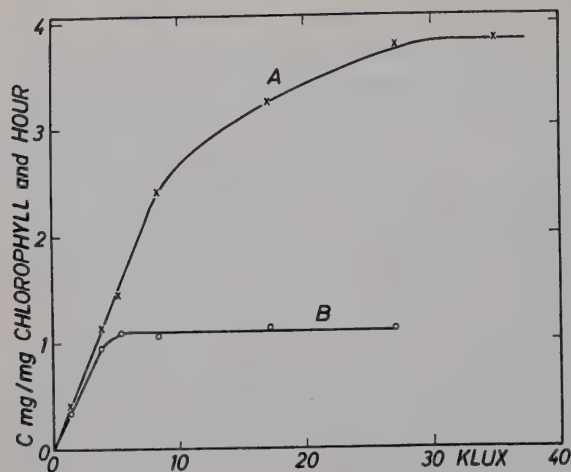


Figure 1. The rate of photosynthesis per unit of chlorophyll $a + b$ as a function of light intensity. *Chlorella vulgaris* grown at 30 Klux (A) and at 3 Klux (B). (1000 lux = 1 Klux).

saturation is nearly 4 times as high in the algae grown at 30,000 lux as in those grown at 3,000 lux.

At 1,000 lux 0.29 mg C per mg chlorophyll $a + b$ is assimilated by the algae grown at the high light intensity and 0.25 mg by those grown at the low light intensity. Recomputed per one cal. per m^2 per second ($0.4-0.7 \mu$), 0.36 and 0.31 mg C are assimilated per mg chlorophyll per hour, respectively.

Both ratios are a little lower than the ratios obtained as a first approximation by Steemann Nielsen and Hansen (1959). The ratio is somewhat higher in the algae poor in chlorophyll grown at the high light intensity compared with the chlorophyll rich algae grown at the low light intensity (cf. p. 870). In the latter the ratio is only 11 per cent higher than the ratio (0.28) found by Gabrielsen in the aurea varieties of the higher plants.

3. Discussion

In the green alga *Chlorella*, chlorophyll $a + b$ must be considered to be the photosynthesis pigments of major importance. It seems reasonable to expect that the importance of the carotenes for photosynthesis is considerably less. In this respect the alga may be compared with the leaves of higher plants. In mixed plankton collected in nature a considerable part of the algae may be species of diatoms, blue green algae and dinoflagellates. In these algae the light absorption by pigments other than chlorophyll may constitute an important part of the energy used in photosynthesis. Finally in the diatoms and the dinoflagellates chlorophyll c is found instead of chlorophyll b .

In plankton collected in nature it is not always appropriate to measure the rate of photosynthesis at low light intensities per concentration of chlorophyll $a+b$ only. We cannot expect that the ratio will be identical with that found when making experiments with a green alga. The rates may be calculated on the basis of at least three other units, 1) the concentration of the total pigments, 2) the concentration of chlorophyll $a+b+c$ and 3) the concentration of chlorophyll a only. Owing to the difficulties at present in obtaining sufficiently exact values of total pigment concentration and even total chlorophyll concentration, the present writer would prefer to use chlorophyll a as a basis. However, we have to wait for special investigations using unicellular cultures of algae belonging to the different taxonomic groups.

The only other alga investigated seems to be a blue green alga. Myers and Kratz (1955) have published simultaneous measurements of the concentration of pigments and the photosynthetic rate in the blue green alga *Anacystis nidulans*. Calculated per one cal. per m^2 per second, 1.2 mg C is assimilated per mg chlorophyll a (the only chlorophyll in blue green alga) and 0.14 mg C per mg chlorophyll + phycocyanin. In *Anacystis* the concentration of phycocyanin is about 8 times as high as that of chlorophyll. If in photosynthesis in incandescent light we arbitrarily assume 4 mg phycocyanin to be equally effective as 1 mg chlorophyll, we may obtain the ratio 0.39 mg C per mg of such pigment unit at one cal/ m^2 second, about the same ratio as found in *Chlorella* using chlorophyll $a+b$ as basis. However, it must be stressed, that this calculation can by no means be taken as a real proof that the photosynthetic efficiency of phycocyanin in incandescent light is just 25 per cent of that of chlorophyll.

If we recalculate the photosynthetic rates per one mg chlorophyll $a+b$ presented in section 2 for *Chlorella vulgaris* grown at either 30,000 or 3,000 lux on the basis of chlorophyll a alone, we obtain the rates 0.49 and 0.42 mg C per mg chlorophyll a per hour at the illumination one cal per m^2 per second (0.4–0.7 $m\mu$).

It is interesting to note that Gessner (1944), in plankton from the eutrophic Wessling Sea in Germany, found that 0.41 mg C is assimilated per mg chlorophyll per hour at 1,000 lux incandescent light (=0.52 mg C at one cal (0.4–0.7 $m\mu$) per m^2 and sec.) Gessner measured chlorophyll a exclusively. Green algae probably predominated in the plankton.

Measurements of the rate of photosynthesis and of chlorophyll a made during July 1957 at the surface for a series of oceanic stations west of Greenland gave as result, that on the average about 0.30 mg C and at the maximum 0.47 mg C is assimilated per mg chlorophyll a per hour at the illumination one cal per m^2 per second (0.4–0.7 $m\mu$, incandescent light). The measurements — unpublished — were made during the cruise of M/S "Dana" by

Mr. Vagn Hansen. In view of the many complications — such as composition of species, presence of possibly dead chlorophyll and presence of possibly inactive chlorophyll due to light inhibition, — it would be unrealistic to expect a better conformity with the values obtained with *Chlorella* during the present investigations. There is every reason to expect a considerably poorer conformity with *Chlorella* perhaps in most cases.

The present photosynthesis experiments were made in incandescent light. In view of the many possible pitfalls for biologists, working with the light dependency of photosynthesis but not being fully familiar with the many aspects of light it may be appropriate to recommend that the experiments are made using incandescent light. It is possible to buy commercial instruments for light measurements of a high standard. These instruments measure the light in lux or in foot candles. These instruments are standardized in incandescent light. Although the units like lux or foot candles have a definite meaning only in connection with the human eye, for comparative work in photosynthesis using the same light source, no risk is present. By a simple conversion factor, lux measured in incandescent light may be converted to an energetic unit (cf. Steemann Nielsen and Hansen 1961).

Instruments for light measurements from different manufacturers may give considerably deviating results if a light source other than incandescent light is used. It would perhaps also be useful to stress, that most instruments are exact only at low light intensities. Therefore, if light of higher intensities has to be measured it is advisable to place a neutral filter before the photocell. The light absorption of the neutral filter must be known.

It is also important that the light meter is constructed so that light received from all angles is measured equally. If the area of the light source is large, it is misleading to use instruments measuring only the rays falling vertically on the surface of the photocell. The light intensity measured in such cases is only a minor part of the effective intensity. The light intensities presented in some well known articles on photosynthesis are quite wrong due to this complication.

Finally we may reemphasize the fact stressed by Gabrielsen 1948, that chlorophyll concentration is a factor of importance only at low light intensities. Theoretically we cannot expect that the rates of photosynthesis at light saturation are dependent on the concentration of chlorophyll. The two curves in Figure 1 illustrate this. However, in plankton of about the same kind grown under identical light- and temperature-conditions the rates of photosynthesis at light saturation may show an apparent dependence on chlorophyll concentration. In definite areas, at definite seasons and at definite depths, the rate of light saturated photosynthesis may be correlated with chlorophyll, if the major part of the latter is not dead or inactive.

Summary

The rate of photosynthesis and the concentration of chlorophyll $a + b$ was measured in *Chlorella vulgaris* cultivated at either 3,000 or 30,000 lux. At the low light intensity one cal/m² × second (0.4–0.7 μ), 0.31 and 0.36 mg C respectively is assimilated per hour per one mg chlorophyll $a + b$.

Compared with the ratios obtained for ordinary green leaves of higher plants, those for *Chlorella* are about 4–5 times higher. However, compared with the ratios for extremely chlorophyll poor leaves — leaves of aurea varieties — those for *Chlorella* are only 11–29 per cent higher. It is shown that the results are in conformity with what is to be expected theoretically.

The ratios to be expected in plankton from Nature are discussed. In addition to the complications due to dead and inactive chlorophyll, the occurrence of other pigments of major importance for photosynthesis is mentioned.

The rate of photosynthesis at light saturation is in itself not a function of chlorophyll concentration. However, in plankton reared under uniform conditions a dependency may nevertheless be found.

Dr. E. G. Jørgensen and the UNESCO stipendiates Mr. C. Teixeira and Mr. A. Magliocca assisted in the performance of the experiments.

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Développement des stomates de peupliers au cours d'une année sèche

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Introduction

L'année 1959 a été caractérisée par un climat extrêmement sec. Le sol de nos pépinières était bien humide au commencement de la saison végétative, mais au cours de la période s'étendant de mai jusqu'au 25 juillet, il a, en raison des précipitations minimales, perdu pour ainsi dire toute la quantité d'eau disponible pour les plants.

Après une courte période de précipitations abondantes, du 25 juillet au 10 août, le restant de la saison végétative fut à nouveau très sec, voir tableau 1.

L'accroissement en hauteur des peupliers adaptés au climat belge suit normalement la courbe d'un S allongé ayant en plein été, l'allure d'une droite.

Au cours de l'année 1959, l'accroissement se ralentit considérablement durant le mois de juillet, et certains clones ont tout à fait arrêté leur croissance et formé des bourgeons terminaux.

Sous l'influence de la précipitation de fin juillet et début août, une reprise brusque de la croissance a eu lieu comme il est montré dans la figure 1.

La grandeur des feuilles développées au cours de la saison 1959 a suivi ces variations, et le but du présent travail est de préciser le développement des stomates sous l'influence de la sécheresse.

Méthode et matériel

La méthode, exclusivement descriptive, appliquée pour caractériser les stomates, utilise leur longueur ainsi que leur nombre par unité carrée de feuille (densité).

Une procédure très simple a été adoptée pour la préparation du matériel neces-

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Tableau 1. *Précipitations au cours de la période de végétation en 1959 et précipitations normales. Grammont.*

Mois	Dates	Précipitations, en mm		
		1959		Normales
		Décade	Mois	
Janvier-avril			232.9	219
Mai	1 — 10	3.8		
	11 — 20	10.8		
	21 — 31	0.3	14.9	57
Juin	1 — 10	3.1		
	11 — 20	1.3		
	21 — 30	18.3	22.7	65
Juillet	1 — 10	2.1		
	11 — 20	0.0		
	21 — 25	0.0		
	26 — 31	62.2	64.3	76
Août	1 — 10	22.0		
	11 — 20	9.3		
	21 — 31	2.5	33.8	75
Septembre	1 — 10	0.0		
	11 — 20	0.0		
	21 — 30	2.5	2.5	60
Octobre	1 — 10	2.5		
	11 — 20	9.7		
	21 — 31	56.6	68.8	70
Novembre			44.3	75
Décembre			98.8	78
L'année: 583.0				775

saire. Des petits morceaux d'épiderme sont prélevés au moyen d'une lame de rasoir sur des feuilles fraîches ou sèches. Les coupes, aussi minces que possible, sont plongées dans l'alcool d'une concentration de 95 % environ jusqu'au moment de l'examen. Elles sont alors déposées dans une goutte d'eau, sur un porte-objet, recouvertes d'un couvre-objet et examinées sous le microscope sans aucune ajoute de produits colorants ou autres.

L'agrandissement utilisé était toujours de 430 ×. La longueur des stomates a été mesurée en utilisant un micromètre avec une unité = 0.0035 mm. Cent stomates par préparation constituent la base de chaque valeur moyenne de longueur.

Pour l'enregistrement du nombre de stomates, tous les stomates visibles dans le champ, sous le même agrandissement, sont comptés. La numération dans 20 champs établit la valeur moyenne, qui correspond à une superficie de 0.098 mm².

La surface des feuilles a été estimée par la méthode de quadrillage (unité 1 cm²).

Sur quatre pousses d'un clone de *Populus nigra* L. (V. 842, provenant de Zutphen, Pays-Bas), la plupart des feuilles, ayant atteint leur développement définitif, ont été examinées à l'état frais. Deux des pousses provenaient de plants greffés, et les deux autres de jeunes boutures de l'année.

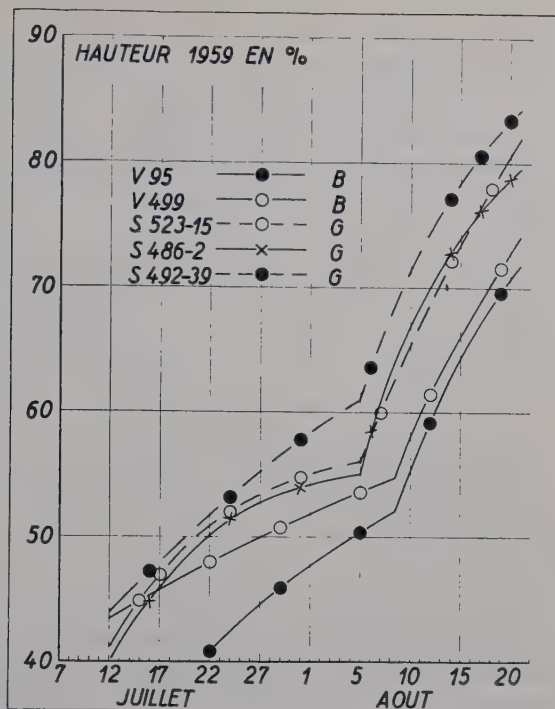


Figure 1. L'accroissement en hauteur de 5 différents clones de peupliers durant les mois de Juillet et d'Août 1959, exprimé en pour-cent de l'accroissement total de l'année. (B: pousse d'une bouture; G: pousse d'une greffe.)

De chaque feuille, deux préparations ont été examinées, avec des résultats concordants, et ce sont leurs valeurs moyennes qui sont représentées ci-après.

En outre, des feuilles d'autres clones, rassemblées en automne, ont été examinées à l'état sec, au cours de l'hiver suivant.

Resultats

Des variations prononcées dans le développement des stomates au cours de la saison végétative, ont été constatées.

Traisons premièrement en détail, la longueur et la densité des stomates du clone V. 842, et ensuite, sommairement, celles des autres clones examinés.

a. *Longueur des stomates.* La variation est démontrée sous forme graphique dans la figure 2. Ici, l'abscisse donne l'ordre des feuilles sur la pousse, de la première à la base, développée au printemps (feuille n° 1) jusqu'à la dernière au sommet de la pousse, formée en automne (n° 61). L'ordonnée à gauche, montre la longueur des stomates, en unités, et celle de droite la surface unilatérale des feuilles, en centimètres carrés.

Les courbes A comportent les données provenant d'une greffe, et les courbes D celles provenant d'une bouture.

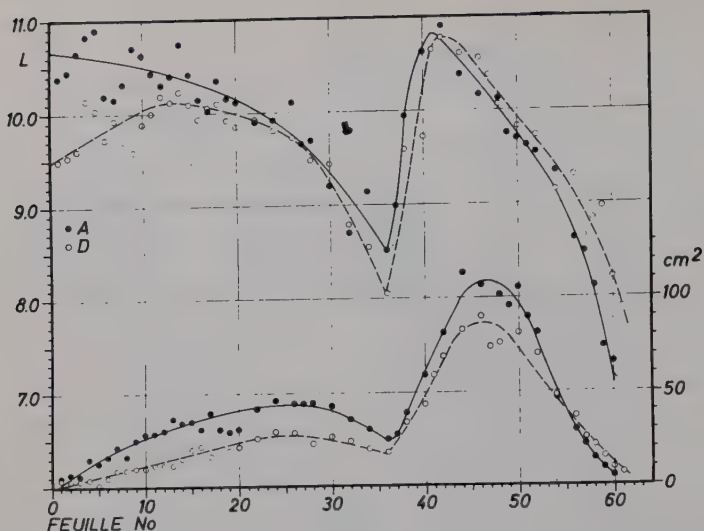


Figure 2. La longueur des stomates durant l'année 1959 chez les feuilles de V.842. A: Feuilles d'une greffe; D: Feuilles d'une bouture. (L'ordonnée à gauche.) La grandeur de mêmes feuilles (l'ordonnée à droite).

Regardons premièrement la longueur des stomates de la courbe A:

Dès le début les feuilles possèdent des stomates d'une longueur presque maximum du clone, mais déjà à partir de la 18ème feuille, la longueur diminue continuellement jusqu'au minimum à la 36ème feuille.

Cette diminution de la longueur correspond à la diminution de l'humidité du sol au cours de la même période, voir tableau 1.

Nous avons pu constater que la 36ème feuille s'est déployée vers le 22 juillet et la 38ème vers le 4 août.

Déjà à partir de la 37ème feuille, un agrandissement de la longueur des stomates a eu lieu et la 38ème feuille a pu former des stomates à peu près normaux quant à leur longueur. Pour les feuilles suivantes, la longueur augmente encore jusqu'au maximum absolu (42ème feuille).

Biologiquement, cette discontinuité dans la courbe s'explique par la forte augmentation des précipitations qui a brusquement changé les possibilités d'un développement normal du plant.

Dès la mi-août, une nouvelle diminution dans la longueur des stomates se présente et elle continue jusqu'à la dernière feuille du plant.

La première partie de cette diminution est exclusivement due à la nouvelle période de sécheresse, commençant mi-août et continuant au mois de septembre.

Etant donné que les dernières feuilles d'une pousse n'atteignent pas un développement complet avant l'arrêt de la saison végétative, ces feuilles ont normalement des stomates petits et, dans notre cas leur développement est, de plus, ralenti par la sécheresse.

En regardant la courbe D, nous constatons qu'elle possède, grosso modo, la même allure. Toutefois, la longueur des stomates des premières feuilles est plus petite que celle de A.

Cette différence s'explique du fait que les feuilles de la greffe sont alimentées d'une façon plus ou moins normale dès le début, tandis que les feuilles d'une bouture sont développées avant que l'enracinement ne soit suffisamment établi pour assurer une alimentation en eau correcte.

Au cours d'une saison normale, la longueur des stomates des boutures, croît jusqu'à ce qu'elle ait atteint la même grandeur que celle des greffes, mais dans notre cas, la sécheresse ralentit l'accroissement et ce n'est qu'après la période des pluies que les stomates de la bouture arrivent à leur grandeur normale.

L'écart-étalon des longueurs du tableau 2, $s_{\bar{x}}$, qui est peu variable d'une préparation à l'autre, a une valeur moyenne égale à 0.10 unité. Les variations des longueurs des stomates ne sont donc pas, dans les domaines importants, dues au hasard.

Quant à la grandeur des feuilles, nous trouvons des variations parallèles à celles constatées pour les longueurs des stomates. Ici également, les premières feuilles de la bouture sont plus petites que celles de la greffe.

E. *Densité des stomates.* Le nombre de stomates par unité de surface, est compté d'une part sur la face supérieure (d_s), d'autre part sur la face inférieure (d_i), et le résultat est montré séparément pour les deux plants A et D dans la figure 3.

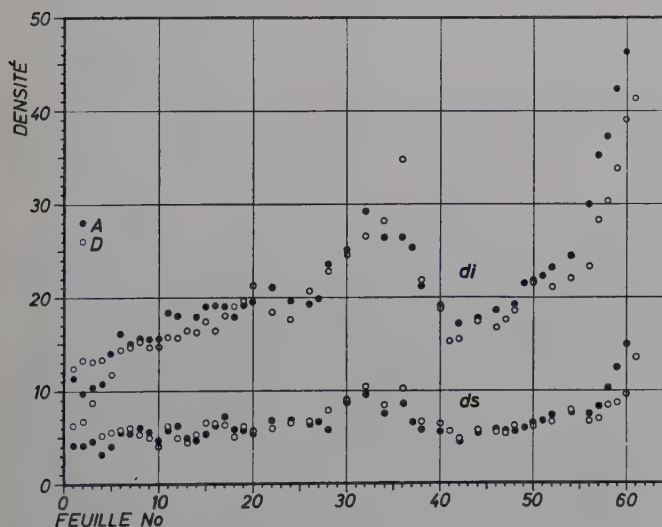


Figure 3. Les densités, d_i et d_s , du clone V. 842 durant l'année 1959.

Des variations considérables de la densité sont également constatées suivant l'ordre des feuilles; mais inverses de celles de la longueur, de telle façon qu'un grand nombre de stomates correspond aux stomates petits et inversement.

Toutefois, à la fin de l'année, la densité des stomates est supérieure à celle constatée sur les feuilles du printemps, même pour une longueur identique des stomates (et une même grandeur de la feuille); question à discuter plus tard.

L'écart-étalon de la moyenne est calculé sur la base de 20 comptages. Sa valeur est donc relativement élevée.

L'écart-étalon de la densité sur la face inférieure des feuilles a une valeur moyenne de 0.62, pour toutes les feuilles sauf les deux dernières, où cette valeur monte à 1.72.

La valeur moyenne de l'écart-étalon de la densité sur la face supérieure est, pour la plupart des feuilles, égale à 0.27;

Pour les feuilles n° 30 à 36, cette valeur monte à 0.46, et sur les dernières feuilles, n° 59, 60 et 61, elle est de 0.55.

Les valeurs importantes de la densité sont donc aussi significativement différentes.

c. *Autres examens.* Les deux autres plants du clone V. 842 ont donné des résultats pareils aux précédents.

La réaction du plant au moment où l'eau revient en quantité suffisante après une longue période de sécheresse est un phénomène général, que nous avons retrouvé chez d'autres types de peupliers au cours de l'année 1959.

Nous avons examiné un bon nombre de feuilles provenant de clones différents, et dans le tableau 2, nous donnons pour 5 de ces clones, un extrait des résultats obtenus; notamment les valeurs moyennes de trois feuilles développées (3 numéros consécutifs):

- a. au printemps,
- b. fin de l'époque sèche,
- c. immédiatement après.

Il ressort du tableau que les 5 clones, provenant d'espèces et d'hybrides différents, ont tous réagi exactement comme le clone V. 842.

L'ensemble des résultats montre clairement l'influence de l'eau sur le développement des feuilles, non seulement quant à leur grandeur mais également quant à la longueur et à la densité des stomates.

d. *Rapports entre les différentes caractéristiques.* Quelques questions se présentent immédiatement, notamment:

1. Quel est le rapport entre la longueur et la densité des stomates?

Tableau 2. Développement des stomates chez 5 clones de peupliers en 1959.

Description	No des feuilles	Dimensions des feuilles en m.m.	Longueur en unités	Densité		Q
				d _i	d _s	
a. feuilles du printemps						
V. 95	14 — 16	60 × 65	9.17	16.9	4.7	28
V. 499	10 — 12	69 × 64	9.32	20.0	7.9	40
S. 523 — 15	13 — 15	93 × 110	9.94	14.2	9.1	64
S. 486 — 2	9 — 11	145 × 158	9.56	17.7	6.8	38
S. 492 — 39	10 — 12	110 × 158	9.79	18.2	3.5	19
b. fin de l'époque sèche						
V. 95	34 — 36	75 × 51	8.26	19.5	6.0	31
V. 499	26 — 28	89 × 79	8.69	22.5	9.3	41
S. 523 — 15	31 — 33	79 × 71	8.90	16.0	11.7	73
S. 486 — 2	26 — 28	125 × 141	8.05	25.6	11.6	45
S. 492 — 39	32 — 34	67 × 84	8.64	28.2	3.8	13
c. immédiatement après						
V. 95	37 — 39	112 × 84	9.25	17.6	5.1	28
V. 499	29 — 31	123 × 116	9.30	20.3	7.7	38
S. 523 — 15	34 — 36	117 × 117	10.38	14.1	9.7	69
S. 486 — 2	29 — 31	168 × 177	9.52	17.7	7.5	42
S. 492 — 39	35 — 38	116 × 118	9.62	17.3	2.3	13

V. 95 et V. 499: boutures de *P. nigra* — S. 523-15: greffe de *P. deltoides* — S. 486-2. greffe de *P. (deltoides × trichocarpa) × P. deltoides* — S. 492-39: greffe de *P. (deltoides × trichocarpa) × P. trichocarpa*.

2. Quel est le rapport entre la densité des stomates sur les deux faces de la feuille?

3. Existe-t'il un rapport entre la longueur des stomates et la grandeur des feuilles?

1. Le rapport entre la longueur et la densité des stomates, établi sur la base de 379 paires de données, prises parmi les feuilles de *P. nigra*, V. 842, est illustré par la figure 4.

Les courbes de la figure reflètent, en somme l'avis général: une densité élevée est concomitante d'une longueur restreinte.

Toutefois, les deux rapports de la figure 4 sont établis sur la base de données très hétérogènes, car les stomates sont développés dans des conditions différentes de climat. En outre, les premières feuilles, provenant des boutures, sont alimentées d'une façon différente de celle des autres feuilles, et enfin, les dernières feuilles d'automne n'ont pas eu un développement complet.

Selon l'avis de l'auteur, la forme des deux courbes reflète surtout l'hétérogénéité du matériel.

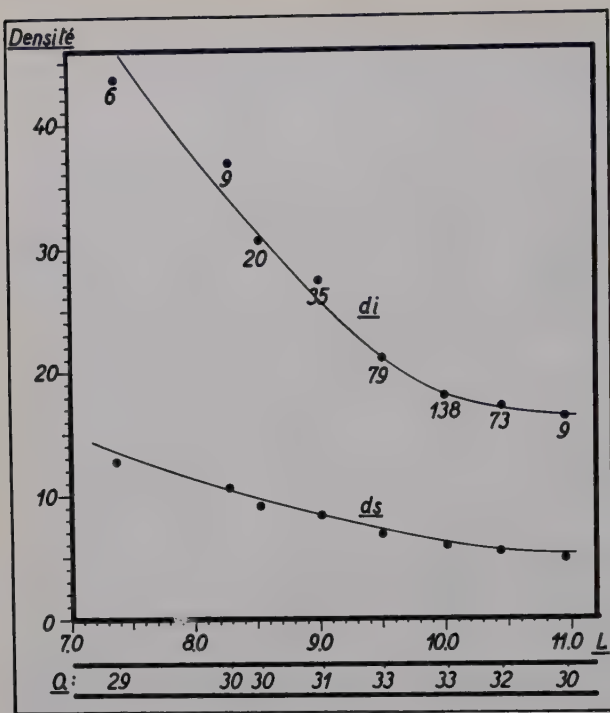


Figure 4. Le rapport entre la longueur des stomates et les densités des stomates, d_i et d_s ; ainsi que le rapport Q y relatif.

2. En établissant le rapport:

$$Q = \frac{\text{densité des stomates dans la face supérieure}}{\text{densité des stomates dans la face inférieure}} \times 100$$

nous constatons qu'il reste constant pour toutes les longueurs de stomates enregistrées.

Cette constatation est en concordance avec nos autres expériences dans ce domaine.

Le rapport Q d'un même clone, reste plus ou moins inchangé quelles que soient les conditions extérieures et, en outre, il semble indépendant du degré de développement de la feuille.

Pour cette raison, le rapport Q caractérise mieux l'individu en question que la densité.

3. En ce qui concerne un rapport entre la longueur des stomates et la grandeur des feuilles, la figure 2 montre clairement, que les deux séries de données varient souvent parallèlement. Toutefois, nous constatons également, qu'une certaine grandeur des feuilles correspond au cours de l'année à différentes longueurs de stomates.

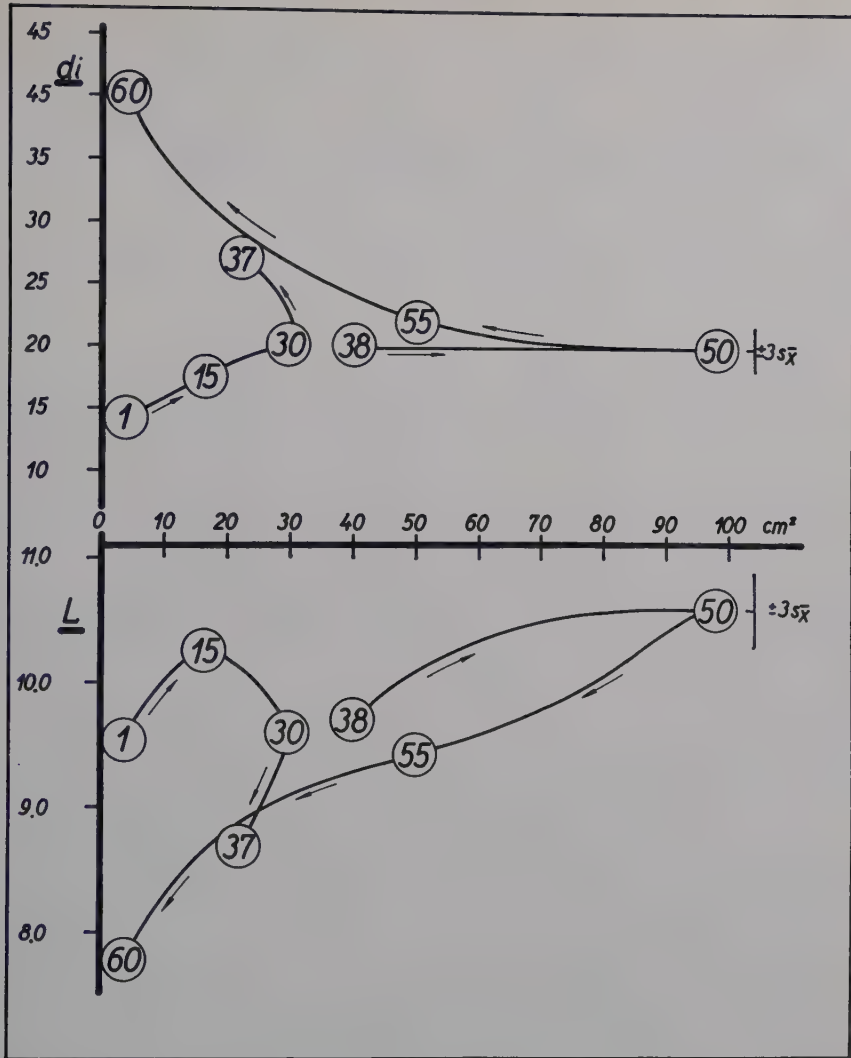


Figure 5. Le rapport, semi-schématique, entre la grandeur des feuilles et la densité d_i (en haut) et celui de la longueur des stomates (en bas), voir le texte.

Nous ne pouvons donc pas mettre en rapport directement ces deux quantités sans indiquer en même temps: l'époque de l'année et les conditions extérieures sous lesquelles les feuilles se sont développées. D'autre part, les différentes sources de variation dans le développement des stomates ne permettent pas d'établir d'une façon simple, les rapports entre la grandeur de la feuille et les caractéristiques des stomates.

Cependant, nous avons trouvé opportun de donner, dans la figure 5, ces deux rapports semi-schématiquement sur la base de toutes les données provenant de V. 842.

Ici, l'abscisse est la grandeur de la feuille, en cm^2 , et l'ordonnée est respectivement la longueur des stomates et leur densité, en unités.

Les rapports sont uniquement indiqués pour les boutures car ceux des greffes sont peu modifiés et suivent le même schéma.

Les chiffres, placés sur les courbes, indiquent l'ordre des feuilles sur la pousse.

Evidemment, les figures reflètent, une fois encore, l'effet des différentes sources de variation:

1. *Longueur des stomates.* Au début, l'influence d'une alimentation d'eau différente suivant le degré d'enracinement. Ensuite, l'influence de la sécheresse de plus en plus accentuée et, après une discontinuité dans le graphique, une courte période où la longueur des stomates est en équilibre et indépendante de la grandeur des feuilles.

Puis, à nouveau, l'influence de la sécheresse se manifeste en diminuant la grandeur des stomates ainsi que celle des feuilles. Cette diminution est encore plus accentuée pour les dernières feuilles du sommet, car leur développement possible est encore retardé en raison de la fin de la période de végétation.

Les variations qui affectent la longueur des stomates sont donc toujours enregistrées sur le graphique en dessous d'un même palier.

2. *Densité des stomates.* On pourrait, à priori, présumer une image inverse de celle des longueurs. Toutefois, nous constatons, que la densité des petites feuilles du printemps est très différente de celle des autres saisons. Les feuilles du printemps ont une densité en dessous d'un certain palier, tandis que toutes les variations au cours de l'été et en automne, se manifestent autour de et au-dessus du même palier.

Il semble donc, que non seulement la sécheresse et l'état du développement influencent la densité des stomates, mais qu'il existe un facteur en plus, dont l'effet ne se manifeste qu'au printemps.

Discussion

Les examens précédents constituent une partie d'une étude sur les stomates chez les peupliers. Les résultats sont, selon l'avis de l'auteur, en bonne concordance avec d'autres observations non encore publiées.

Une discussion approfondie est actuellement prématurée, et nous nous limi-

terons à quelques considérations concernant les variations des longueurs et des densités des stomates en général.

A priori, on peut admettre que les caractéristiques des stomates (la longueur et la densité des deux surfaces de la feuille) sont déterminées par la constitution héréditaire des plants d'autant plus qu'il ressort de la littérature, que ces données varient souvent fondamentalement d'une espèce à l'autre.

Etant donné qu'il s'agit de caractères héréditaires d'ordre quantitatif, le développement de la longueur et de la densité est très influencé par les conditions du milieu, et principalement par la présence ou l'absence de l'eau.

Salisbury (1927) souligne la nécessité d'employer des feuilles bien comparables, mais étant donné que la variation est déterminée par un complexe de facteurs, il est souvent difficile de savoir lequel des facteurs a l'effet dominant dans un certain cas.

En tenant compte de l'ordre des feuilles sur la pousse, nous avons une possibilité supplémentaire d'éviter la subjectivité de nos estimations.

Les examens précédents démontrent premièrement l'importance de l'eau pour un développement normal des stomates, ce qui est d'ailleurs déjà bien accepté depuis longtemps.

Il est important de retenir avec quelle vitesse la longueur et la densité peuvent changer d'une feuille à l'autre sous l'influence de l'eau, ce qui confirme la conclusion de Salisbury (1927 p. 4): «It is suggested that the humidity of the internal and external environments of a leaf are the controlling factors in determining the numerical frequency of its stomata».

Cette conclusion a un aspect général, tandis que la loi de Zalensky ne semble qu'exprimer un parallélisme entre la position des feuilles sur la tige, et les conditions plus ou moins xéromorphiques. Selon la loi de Zalensky, les feuilles de la moitié supérieure de la tige diffèrent toujours de celles de la partie inférieure, quant à la dimension des feuilles et des cellules épidermiques ainsi que des autres caractéristiques (Maximov 1938).

Les courbes précédentes démontrent clairement que la position en soi n'indique pas grand chose quant à la longueur et la densité des stomates sauf dans les cas où les conditions du milieu ont changé durant la saison végétative.

Nous avons, à plusieurs reprises, parlé du degré de développement de la feuille, et nous entendons par là le rapport de ses dimensions actuelles à celles qu'elle atteindra à l'état adulte.

Il s'agit donc d'une expression relative, qui n'a aucun rapport avec la grandeur de la feuille.

Nous n'avons pas examiné, au cours de l'étude présente, l'influence du degré de développement de la feuille, étant donné que, sauf pour les feuilles

extrêmes au sommet du plant, les déterminations ont porté sur des feuilles arrivées à leur état définitif.

Toutefois, selon nos autres examens, cette source de variations est extrêmement importante, et elle peut masquer complètement l'effet des influences du milieu. En réalité, l'influence du milieu arrête le développement de la feuille à un certain stade, mais si on examine des feuilles à peine déployées, on n'enregistre pas l'effet du facteur recherché.

Plusieurs auteurs ont signalé, depuis longtemps, qu'il y a un rapport entre le nombre et la longueur des stomates (e.a. Weiss 1865, Muenscher 1915). Cette généralisation a été établie par comparaison des données provenant des espèces différentes, et elle donne l'impression d'une corrélation négative.

Comme la figure 4 le démontre, une telle corrélation négative entre la longueur et la densité des stomates est également valable quand il s'agit d'un même clone.

Chez les feuilles jeunes d'un développement faible, nous trouvons des stomates d'une longueur restreinte, tandis que les feuilles adultes, qui se sont épanouies sous des conditions de milieu favorables, possèdent des stomates ayant une longueur normale pour l'individu en question.

Pour cette raison, il faut considérer que les courbes de la figure 4 en réalité reflètent l'hétérogénéité des données.

Les graphiques de la figure 5 semblent également confirmer ce raisonnement.

Toutefois, la faible densité observée sur les feuilles du printemps, ne peut s'expliquer par ce raisonnement.

Actuellement, nous ne possédons pas d'autres observations qui peuvent donner l'explication; mais elle se trouve peut-être dans un certain dimorphisme des feuilles, similaire à celui que Critchfield (1960) a constaté chez *Populus trichocarpa*.

Finalement, il est utile de signaler que le nombre des stomates, visibles à l'état définitif, augmente au cours de l'épanouissement de la feuille. Les comptages et les mesurages ne portent pas sur les cellules mères mais uniquement sur les cellules stomatiques ayant la fente caractéristique.

Si la superficie d'une feuille augmente, disons de 10 à 100 cm², nous ne retrouvons qu'une diminution de la densité de 38 à 19 par unité carrée.

C'est-à-dire que la petite feuille ne possède qu'un cinquième du nombre des stomates de la grande feuille.

Un résultat similaire est obtenu en faisant la comparaison la plus défavorable: la densité maximum absolue d'une petite feuille de 5 cm², soit $d_i = 55$ et $d_s = 19$, et la densité minimum absolue d'une grande feuille de 113 cm², soit $d_i = 13$ et $d_s = 3$.

On peut en déduire que la formation des stomates à l'état définitif se passe

assez tard durant le développement et qu'elle suit le rythme d'épanouissement de la feuille.

Résumé

Le développement des stomates de peuplier a été examiné durant la saison végétative de 1959. Cette année se caractérise par un climat extrêmement sec.

Des variations prononcées dans la longueur ainsi que dans la densité des stomates ont été constatées en relation avec la quantité d'eau disponible pour les plants, de telle manière qu'une époque sèche provoque une diminution de la longueur, en même temps qu'une augmentation de la densité.

L'arrivée abondante d'eau après une période sèche provoque un changement brusque — d'une feuille à l'autre — dans les grandeurs et les densités des stomates.

Une densité élevée est concomitante d'une longueur restreinte. Toutefois, la forme des courbes de cette figure reflète surtout l'hétérogénéité du matériel, donc l'influence de l'environnement.

Le rapport Q entre les densités des stomates des deux faces semble être indépendant du degré de développement de la feuille.

La formation des stomates à l'état définitif se passe assez tard durant le développement et suit le rythme d'épanouissement de la feuille. Sous des conditions normales, la grandeur de la feuille n'influence pas la grandeur et la densité des stomates.

Tout de même, il semble qu'il existe un facteur en plus dont l'effet ne se manifeste qu'au printemps, car la densité des stomates est inférieure, à cette époque, à celle mesurée durant le reste de l'année.

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Photoreactivation at 223 m μ in *Platymonas*

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Introduction

The action spectrum of photoreactivation in the motility of the green alga *Platymonas subcordiformis* has recently been determined between 308 and 500 m μ (Halldal 1961). This action spectrum curve showed a close resemblance to the absorption spectrum of a flavoprotein (Corran *et al.* 1939), and the conclusion was drawn that a flavoprotein was involved in the photo-recovery of one type of photoinactivation where injury evidently was caused to the desoxyribonucleic acid (DNA) synthesis. These deductions were based upon action spectra analyses where the biological effects of the radiations were studied one week after the exposure. After this period of time it was not possible to record any damaging effect of the 223 and 238 m μ radiation. These wavelengths were, however, very effective in the immediate immobilization of the algae, and the action spectrum determination of this auto-reversible photoinactivation indicated a damage to proteins. The purpose of the present investigation was to study the effect of radiation between 223 and 313 m μ in the *photorecovery process* after the cells had been injured with 265 m μ radiation. As the radiation at 223 and 238 m μ did not cause damage to the delayed reduction in motility, these wavelengths should be particularly suited for studies of the protein portion of the presumable flavoprotein chromophore involved in photoreactivation. With irradiation at these wavelengths one would expect one of two possible reactions to take place: (1) *Photoinactivation* by the destruction of the protein component of the chromophore. This reaction would be reflected if photoreactivation, for instance with blue light, could be inhibited by simultaneous irradiation at wavelengths between 220 and 238 m μ .

(2) *Photoreactivation* by an activation of the protein component of the chromophore. This could possibly take place through an energy transfer from the protein part to the flavin fraction. It will be shown in the present publication that the latter of the two possibilities mentioned above took place, as the effect of the 223 m μ radiation in photoreactivation surpassed the effects at all other wavelengths tested.

Material and Methods

The unicellular Volvocales *Platymonas subcordiformis* (Wille) Hazen (Gibor's strain) has been used in these experiments. By the use of dilute samples, where the inactivation was performed in a sample contained in a cuvette which was continuously rotated when exposed to the 265 m μ radiation from a Bausch & Lomb monochromator, a more uniform treatment was achieved than in earlier experiments (Halldal 1961). The value selected for the action spectrum plot was also lowered to 10 per cent reactivation in the present investigation. These applications made it possible to get reliable values for action spectrum determinations at only a fraction of the intensities used earlier. The methods followed are only modifications of those outlined in an earlier investigation (*l.c.*), and therefore they will be only briefly summarized here. All irradiations were performed with the Bausch & Lomb grating monochromator. Inactivation was performed at 265 m μ . A sample which was contained in a crystal quartz cuvette was rotated 120 times per minute and irradiated from below by the use of a surface aluminized mirror for 30 minutes with 8×10^{12} quanta/cm² · sec. After this irradiation the motility in the sample was reduced from 80 per cent cells in motion to about 10 per cent on the seventh day with no photoreactivation. Immediately after this exposure the dial of the monochromator was turned to the desired wavelength and the sample after-irradiated for two hours with about 20×10^{12} quanta/cm² · sec (adjusted with the Vee-slide of the exit slit and accurately determined in each case with a calibrated RCA 1P 28 photomultiplier tube). A modification had to be applied at 223 m μ (see below). At zero time after-exposure, and every half hour thereafter a small sample was removed from the cuvette with an adjusted syringe and transferred to 5 ml of medium in culture tubes with glass covers. Following this they were placed in the dark for at least 12 hours, and then allowed to grow and develop in growth cabinets with light between 500 and 800 m μ for one week. This light was shown to be ineffective in photoreactivation. The per cent of cells in motion was then recorded and the values plotted for each wavelength tested against incident numbers of quanta. The reciprocals of the numbers of quanta that gave a photoreactivation of 10 per cent were then plotted against wavelength of radiation, which then represent the action spectrum of photoreactivation. The following wavelengths were tested: 223, 238, 302, 313 m μ and 365 and 436 m μ in order to get check points on the action spectrum curve determined earlier (Halldal 1961). As both the photoreactivating effect at 223 m μ and the lower energy output of the monochromator at this wavelength were of different orders of magnitude than at longer wavelengths, the relative effect of the 223 and the 313 m μ radiations has been tested both for the effect produced by the same numbers of incident quanta per unit time, and the quanta required to give the same photoreactivating effect within a certain fixed time.

Results

In Figure 1 is shown the effect of radiation at different wavelengths in photoreactivation. A pronounced photoreactivation was observed at 223 m μ . At 238 m μ a small reactivation was recorded immediately after the start of the exposure, but prolonged excitation produced inactivation. This is an indication that the 238 m μ region is at the transition between photoinactivation of the DNA synthesis and the photoreactivation of the same. Between 238 and 289 m μ photoinactivation occurred (Halldal 1961). At 302 m μ photoreactivation was demonstrated with moderate efficiency. 313, 365, and 436 m μ represent check points in order to enable a comparison with the action spectrum curve published earlier. When the relative effect of the 223 m μ radiation was tested against that of 313 m μ , it was shown that the numbers of quanta required of the 313 m μ radiation to give the same degree of photoreactivation over the same period of time were five times those of the 223 m μ radiation. This is in agreement with the data presented in Figure 1.

Figure 2 gives the action spectrum of photoreactivation between 223 m μ and the longest wavelength where photoreactivation was observed, at 480 m μ . The curve from 308 to 500 m μ is a reproduction of the curve published earlier, and the points on the curve at 313, 365, and 436 m μ represent the results from the controls taken during the present investigation. Below 308 m μ a rapid drop was recorded. A clear photoreactivation occurred at 302 m μ , but radiation at 289 m μ produced inactivation. In Figure 2 has also been included the absorption spectrum of a flavoprotein taken from the literature (Corran *et al.* 1939). At all wavelengths where photoreactivation was demonstrated good agreements exist between these two curves.

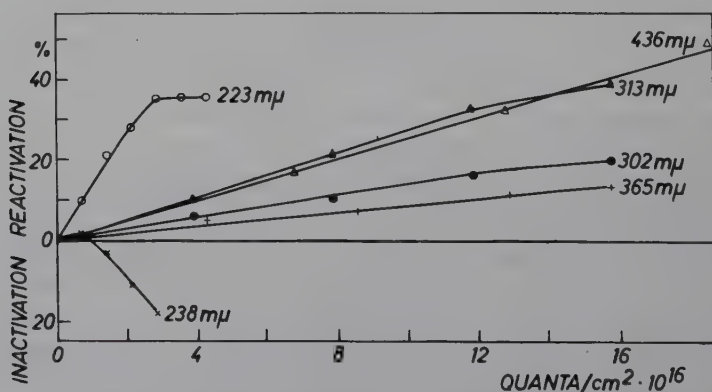
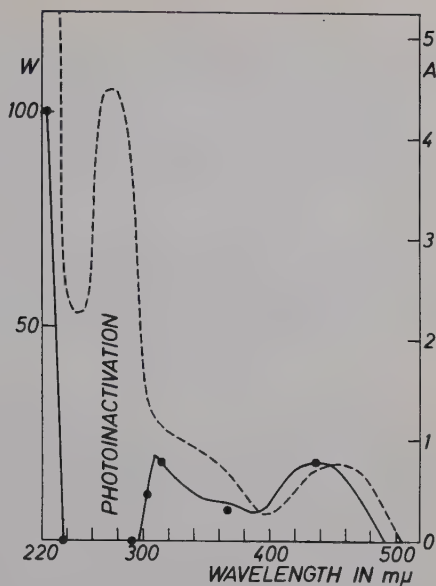


Figure 1. The percentage of photoreactivation and photoinactivation at different wavelengths plotted against dose of radiation.

Figure 2. Action spectrum of photoreactivation in motility of *Platymonas* recorded on the seventh day after irradiation (—, left ordinate); and the absorption spectrum of a milk flavoprotein (from Corran *et al.* 1939, . . ., right ordinate). For further explanation about the action spectrum plot see text.



Discussion

In the earlier investigation it was concluded that a flavoprotein was the radiant energy absorber in photoreactivation in the delayed reduction in the motility of *Platymonas*. This conclusion was based entirely upon action spectrum determination between 308 and 500 m μ . In this region the action spectrum curve showed a better agreement with a flavoprotein absorption curve than with the other candidating pigments (carotenoids and pterins). The effect with the 223 m μ radiation strongly supports this hypothesis.

The action spectra determinations that have been performed on photoreactivation indicate that different chromophores are involved in different biological systems (see Jagger 1958, Tagueeva and Dubrov 1961). It seems therefore difficult to obtain a unified explanation for photoreactivation. Some steps in this reaction can, however, be assumed to be common. Rupert *et al.* (1957) showed that a portion of *Escherichia coli* extract active in photoreactivation *in vitro* was separable into a dialyzable, heat-stable and a non-dialyzable, heat-labile fraction. These two fractions lost their effect upon separation, and the effect was restored when they were recombined. Rupert *et al.* did not draw any conclusion about the nature of these two fractions. It is reasonable to assume that a similar system is involved in the photoreactivation in *Platymonas* where an enzyme seems to be involved which carries a flavin as the prosthetic group. This assumption requires an energy transfer

from the protein fraction to the flavin part. One of the requirements for such energy transfer is that the emission spectrum of the donator overlaps with the absorption spectrum of the acceptor (Bücher 1953). When irradiated in the far ultraviolet some proteins containing aromatic amino acids have been shown to emit radiation in the spectral region where oxidized riboflavin absorbs (Shore and Pardee, 1956 a), and Shore and Pardee (1956 b) also showed that energy transfer occurred in some artificial dye-conjugates of proteins. These findings show that an energy transfer from the protein to the flavin part is theoretically possible under the assumption that the two substances are not too far apart. According to calculations this distance should be 20–35 Å (see Förster 1960).

In an earlier publication (Halldal 1961) it was pointed out that the commonly accepted definition of photoreactivation by Jagger (1958) "*Photoreactivation is the restoration of ultraviolet radiation lesions in a biological system with light of wave length longer than that of the damaging radiation*" needed revision, as it was demonstrated that radiation between 500 and 800 m μ completely restored the photoinactivation of immediate immobilization at 223 and 238 m μ in *Platymonas*. The reversal took place only to some degree in the dark. This photoeffect had, however, nothing to do with photoreactivation, as it evidently was restricted to metabolism in general driven by photosynthesis.

In the present investigation another disagreement with the definition of photoreactivation has been demonstrated, as it was shown that radiation lesions to the DNA metabolism can be repaired with radiation of *shorter* wavelengths than that of the damaging radiation. It remains to be shown whether photoreactivation at these shorter wavelengths commonly takes place, or if the delayed reaction in the motility of *Platymonas* is unique in this respect. Because the reaction in *Platymonas* may represent an exception, no attempt will be made at this stage to introduce a new definition of photoreactivation. However, it now seems possible to restrict the spectral regions where the different photoeffects are observed much better than by the use of unprecise and unsatisfactory expressions like *ultraviolet radiation* and "*light*" of longer wavelength.

Summary

The action spectrum of photoreactivation of the delayed reduction in motility of the green alga *Platymonas subterdifformis* has been determined with particular emphasis on the far ultraviolet region in order to study the protein part of the flavoprotein which is assumed to be involved in the reac-

tion. A pronounced photoreactivation occurred at 223 m μ which is at shorter wavelength than that of the damaging radiation. Between 238 and 289 m μ photoinactivation was evidently caused to DNA metabolism, while photoreactivation resumed at 302 m μ . Between 308 and 500 m μ the curve had been determined earlier. It is assumed that the radiant energy absorbed by the protein part is transferred to the flavin fraction.

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Effect of Filtrates from Cultures of Unicellular Algae on the Growth of *Staphylococcus aureus*

By

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Introduction

The production of antibiotic substances by algae has been demonstrated by several authors. Auto-inhibition of the growth by products accumulated in the nutrient solution was found in cultures of the following algal species: *Nostoc punctiforme* (Harder 1917), *Chlorella vulgaris* (Pratt and Fong 1940), *Skeletonema costatum* (Levring 1945), and *Nitzschia palea* (Denffer 1948). Inhibition of the growth of some algae by substances produced by other algae was observed by Lefevre *et al.* (1949–1952), Harder and Oppermann (1953), Rice (1954), Jørgensen (1956 and 1957), and Proctor (1957). Antibiotic activity of substances produced by algae on the growth of certain bacteria was demonstrated by Pratt *et al.* (1944), Spoehr *et al.* (1949), Blaaw-Jansen (1954), Steemann Nielsen (1955), Emeis (1956), Sieburth (1959), and Jørgensen (1962).

Still very little is known about the nature, the formation and the action of the antibiotic substances formed by algae. The substance most intensively studied is the so called "chlorellin" discovered by Pratt and thoroughly studied by Pratt *et al.* (1944) and Spoehr *et al.* (1949).

The present paper presents the data from a number of experiments involving tests of cell-free filtrates from cultures of various unicellular algae. A couple of algal species have been used in the experiments but the most easily interpreted results were found by the use of *Chlorella vulgaris* (strain

no. 211/11 h from the Cambridge Culture Collection). Because of the authors' special interest in the action of the antibacterial substances in natural waters the experiments were made exclusively with non-concentrated filtrates of algal cultures.

In connexion with the experiments described in this paper the junior author has carried out experiments in which extracts of cells and of culture solutions were tested on *Bacillus subtilis* (Jørgensen 1962). During this investigation it was found that both cell extracts and extracts of culture solutions contained chlorophyllide which in a photo-oxidative form inhibited the growth of *Bacillus subtilis*.

Material and Method

Strain 209 P of *Staphylococcus aureus* was used as test organism, this being the organism commonly used in penicillin tests. It was supplied through the courtesy of Dr. Martin Kristensen, The State Serum Institute, København.

Besides the above mentioned strain of *Chlorella vulgaris* strains of the following algal species have been used in the experiments: *Chlorella pyrenoidosa*, *Scenedesmus quadricauda*, *Anabaena cylindrica* and *Nitzschia palea*.

The cultures were started from bacteria-free stock cultures but the method of cultivation used at the start of the investigation did in no way ensure that the algae remained bacteria-free throughout cultivation. However, the experiments illustrated in Figures 2 and 3 were absolutely free of bacteria.

In most cases the modification of Österlind's *Scenedesmus* medium described by Jørgensen in 1962 was used as culture medium. The algae were grown either in gaswashing bottles or in a special pipette container constructed by the junior author. In the latter the culture is aerated from the bottom and the algae do not settle as is usually the case in gaswashing bottles. The pipette container is shown in Figure 1. It is constructed from a common, commercially available pipette, the

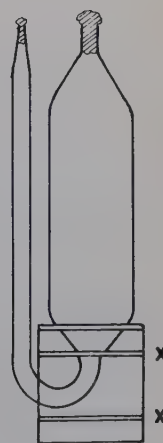


Figure 1. Pipette container for cultivation of algae in its plastic stand.
×-× = two rubber bands fastening the plastic stand onto the pipette container.

upper tube of which is cut off at a small distance above the broad part of the pipette. The remaining part of the upper glass tube is used for closing the algal container with cotton. The algal culture is contained in the broad part of the pipette and the lower glass tube is bent as shown in the Figure. The aeration of the culture is made through this tube. This end of the tube is also closed with a cotton plug. The stand for the pipette container is made from a plastic container, the major part of its bottom having been removed with only a narrow rim left. When the plastic container is placed upside down its rim fits the lower conical end of the algal container. A slit is made in the side of the plastic container to introduce the bent tube for aeration.

The cultures were always aerated with air containing 5 % CO_2 . The cultures were grown in a water bath with continuous illumination from fluorescent tubes (about 4000 lux) and at a temperature of 20°C.

The cell-free algal culture solutions used in the tests as well as the algal medium used for the controls were always prepared by filtration through a Seitz filter.

Test Technique I. The algal culture solution was tested in test-tubes. To 5 ml of Penassay Broth (after Difco Manual) was added 5 ml of Seitz-filtered algal culture solution (or Seitz-filtered fresh culture medium for the control), sufficient phosphate buffer to give a pH of 6.5 and a certain definite volume of a freshly prepared suspension of *Staphylococcus aureus*. Four test tubes were used in each series. The incubation of the bacteria was carried out in a thermostate at 37°C.

The growth of the bacteria was determined by turbidimetric measurements using a Klett-Summerson Photoelectric Colorimeter with a blue filter. The turbidity of the broth + the test tubes was measured at the start of the experiment. These values were subtracted from the values measured at different times during the experiment to give the growth of the bacteria.

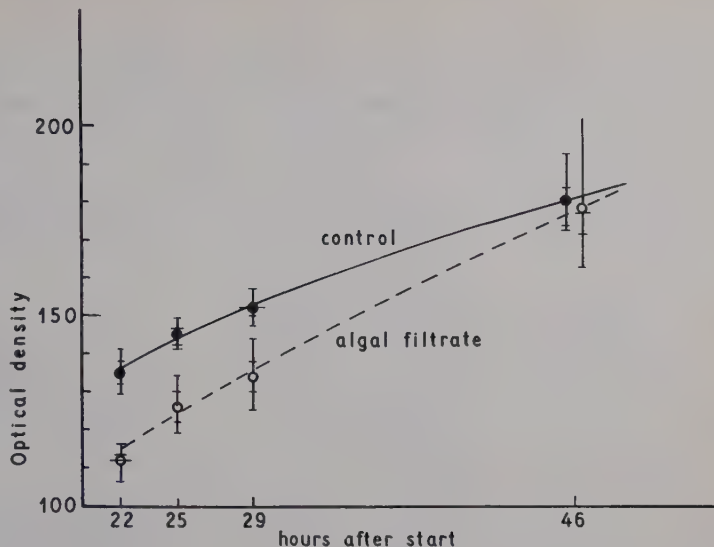
Test Technique II. By this technique the tests were carried out in test tubes in broth as in the above mentioned technique but the incubation took place at room temperature. After 24 hours of incubation three different dilutions were made of the bacteria suspension in each test tube and 1 ml of each dilution inoculated on agar plates and counted after 24 hours of incubation at 37°C.

Experiments

The experiments presented in Figures 2 and 3 were carried out during the junior author's stay at Carnegie Institution of Washington, Department of Plant Biology. Both cultures of *Chlorella vulgaris* used in the experiments were one month old when they were tested. They were grown at different times in a shaking apparatus at a continuous illumination of about 2000 lux and at a constant temperature of 20°C. They were not aerated. The cultures were found to be bacteria-free by testing on two different media. Test Technique I was used for these two experiments.

The experiment presented in Figure 2 shows that the filtrate from the algal culture contains a substance which inhibits the growth of *Staphylococcus*, 22, 25 and 29 hours after the start of the experiment but the effect

Figure 2. The growth of *Staphylococcus* in broth with the addition of fresh algal medium (control) and in broth with the addition of filtered culture solutions from a 1 month old culture of *Chlorella vulgaris*. The horizontal lines represent single determinations, the circles the average values of four determinations.



of the substance is waning and after 46 hours no difference is found between the control and the sample of the algal filtrate.

The experiment in Figure 3 shows very clearly that the filtrate from the algal culture contained a substance which accelerated the growth of *Staphylococcus*. 5 and 17 hours after the start of the experiment no significant difference was found between growth in the two series but after 40 hours there was a clear difference.

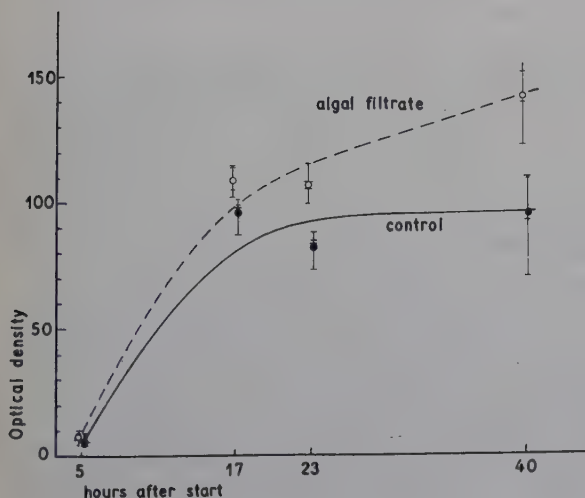


Figure 3. An experiment similar to that of Figure 2 but carried out with another culture of *Chlorella vulgaris* of the same age.

Table 1. *The variation in the action of the growth affecting substances in filtered culture solutions of Chlorella vulgaris in a number of experiments.* The figures represent average values of three bacteria counts. The figures in brackets show the variation in three plates. Inhibition was observed in 9 experiments, acceleration in 4.

I		II	
Age of culture in days	Number of Staphylococcus in control	Number of Staphylococcus in test of algal filtrate	in % of I
11	100 (78— 124)	8 (0— 17)	8
11	830 (474—1082)	208 (192—230)	25
3	462 (320— 661)	134 (58—197)	29
10	18 (133— 212)	55 (38— 74)	31
6	52 (31— 65)	20 (7— 33)	38
6	487 (458— 516)	221 (198—242)	45
6	292 (277— 314)	177 (145—201)	61
7	50 (45— 58)	33 (22— 45)	66
10	687 (668— 721)	509 (499—521)	74
11	69 (32— 91)	114 (101—130)	165
7	69 (65— 78)	145 (103—178)	210
8	83 (79— 91)	302 (259—327)	364
7	19 (12— 28)	425 (352—475)	2240

A vast number of experiments have been carried out in Copenhagen in order to study the action of substances in the algal cultures on the growth of Staphylococcus. We have tried to grow the algal culture used for the experiments under the most homogeneous conditions but have always found great variations in the action on Staphylococcus of the various filtered algal cultures.

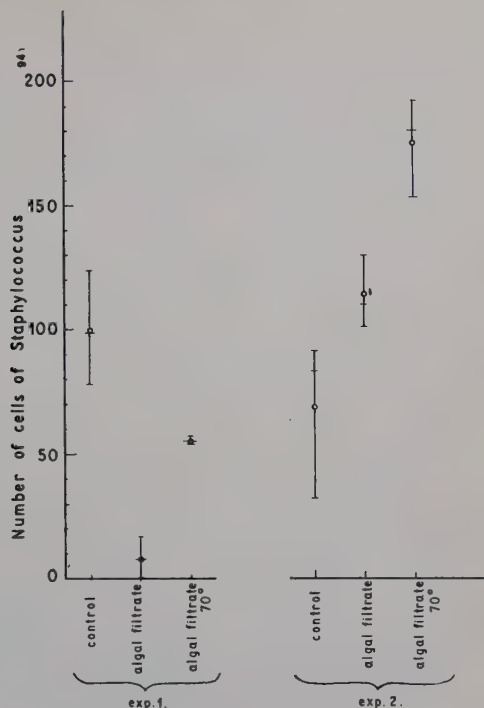
A number of experiments in which test technique II was used for the determination of bacterial growth shows this clearly (Table 1).

The age of the tested algal cultures varied from 3–11 days. It is seen from Table 1 that in some cases an inhibition of the growth of Staphylococcus was found, in other cases an acceleration of the growth. Furthermore the degree of the inhibition or acceleration varied very much. The experiments suggest that both growth inhibiting and growth accelerating substances are found in the Chlorella cultures and that which one of them is present in the greatest quantity depends on factors as yet unknown.

All of the following experiments were carried out with test technique II. Attempts were made to separate the inhibiting and accelerating substances.

Heating of the algal filtrate. Figure 4 illustrates two different experiments in which the filtered algal solution was heated to 70°C for 20 minutes, then cooled again to room temperature and tested in broth on Staphylococcus. In experiment 1 the algal filtrate gave a clear inhibition of the growth of

Figure 4. Two different experiments both carried out with 10 days old cultures of *Chlorella vulgaris*. The filtered culture solution was heated at 70°C for 20 min.



Staphylococcus. The heated algal filtrate also gave some inhibition but to a much lesser extent than did the untreated filtrate.

In experiment 2 the algal filtrate caused an acceleration of the bacterial growth, and the heated algal filtrate increased this acceleration considerably.

The two experiments suggest that both growth inhibiting and growth accelerating substances are present in the algal filtrate. The heating destroys some but not all of the inhibiting substances. In experiment 1 some inhibition was still found after the heating.

Illumination of the algal filtrate. In the experiment shown in Figure 5 the algal filtrate was illuminated for 1 hour before the test with an incandescent lamp giving 1500 lux. The untreated algal filtrate caused slight inhibition of the bacterial growth while the illuminated algal filtrate gave a considerable acceleration of the growth. Thus part of the inhibiting factor was probably inactivated by illumination.

Illumination and Norit-treatment of the algal filtrate. Figure 6 presents an experiment involving illumination and percolation through a column of Norit (a preparation of activated carbon) of an algal filtrate. The untreated algal filtrate caused slight inhibition of the bacterial growth. Illumination

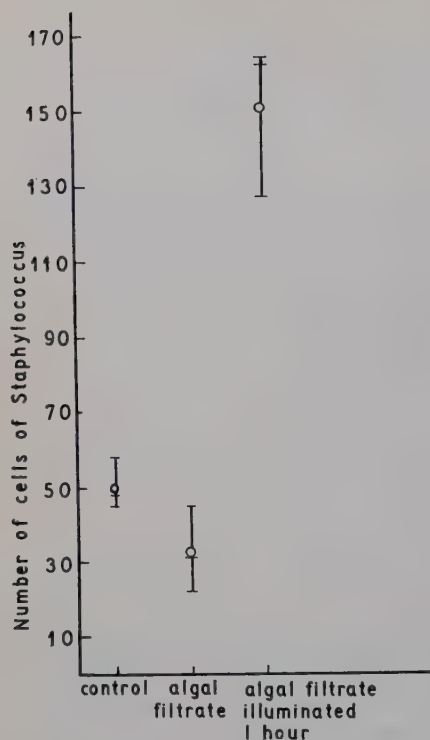


Figure 5. Experiment similar to that of Figure 4 with a 7 days old *Chlorella* culture in which the algal filtrates were illuminated for 1 hour before testing.

of the algal filtrate neutralized the effect. The growth of the bacteria did not differ from that of the control. The effect was found to be the same whether the duration of the illumination was 30 minutes, 1 hour or 2 hours.

When the untreated algal filtrate was percolated through a column of Norit the bacterial growth was found to be accelerated but at the same time a very great variation was found in the three samples from the same series. When the algal filtrates, illuminated for 30 minutes, and for 1 hour, respectively, were percolated through a Norit column the result was found to be the same as in the case of the untreated algal filtrate. After percolation through a Norit filter the algal filtrate illuminated for 2 hours caused a very considerable acceleration of the growth of *Staphylococcus*.

The experiment suggests that at least two different inhibitors are present in the algal filtrate in the experiment illustrated in Figure 6. One inhibitor is inactivated by illumination. Another is absorbed to the Norit.

Tests on slow growing bacteria. In the above mentioned experiments the algal filtrates were tested on bacteria comparatively fast growing. In the experiments described by Emeis (1956), who used *Escherichia coli* as test

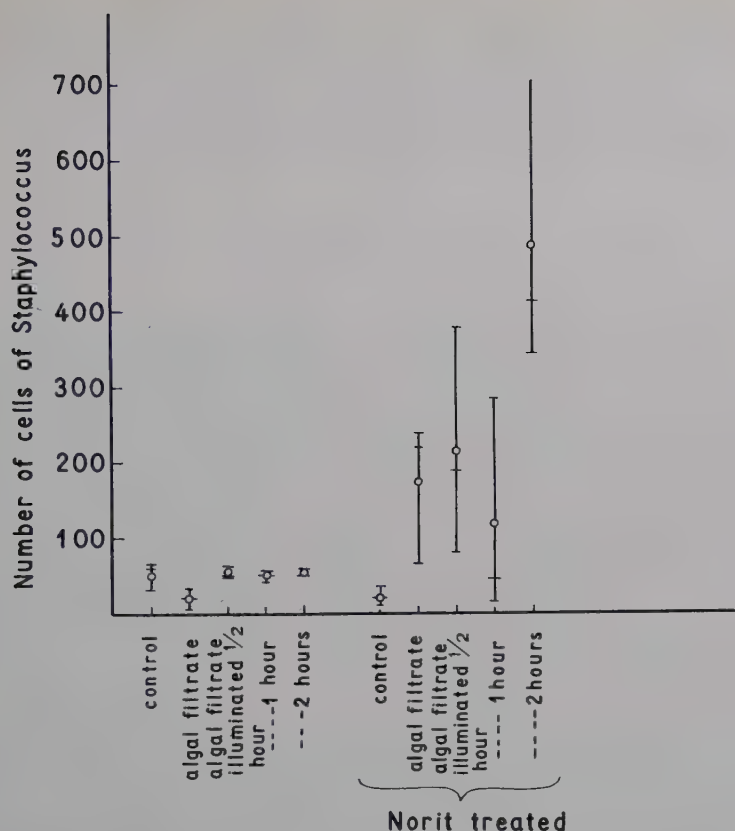


Figure 6. Experiment similar to those of Figures 4 and 5 with a 6 days old *Chlorella* culture, in which the algal filtrate was either illuminated or percolated through a column of Norit or was first illuminated and then percolated through a column of Norit.

organism, the influence of the filtrate was examined in a saline solution in which this organism does not grow. Emeis got a very pronounced anti-bacterial effect by using this test technique.

In the experiments described by Emeis we have been most interested in the finding that the number of bacteria cells was considerably reduced when a suspension of *Escherichia coli* was filtered through an illuminated algal layer of *Nitzschia palea* but that this was not true when the algae were not illuminated. The reduction in the number of bacteria was much greater 24 hours after the filtration than after 3 hours.

Repeated attempts to reproduce the experiments of Emeis in this laboratory have not been successful.

If, nevertheless, it is true as Emeis' experiments appear to suggest that slow growing bacteria are more sensitive to antibiotics than are fast growing ones, then this is very interesting in view of the fact that ordinary sea water bacteria not attached to surfaces show only slight growth and may thus be strongly affected by growth inhibiting substances.

Discussion

In the experiments described above non-concentrated algal culture solutions were used. In other experiments by the junior author (Jørgensen 1962) extracts of cells as well as of culture solutions were tested on the growth of the bacteria *Bacillus subtilis*. The extracts were applied to strips of filter paper, paperchromatograms were made and the paper chromatograms were tested on agar plates with *Bacillus subtilis*. Two different derivatives of chlorophyll from cell extracts were found to have inhibiting effects on the growth of the bacteria. One was chlorophyllide. It has no effect when tested in the dark but when illuminated it is converted into a growth inhibiting substance. In addition some growth inhibiting substances were found which had no relation to the plant pigment and which appear to be different substances in the three algal species tested.

The chlorophyll derivatives with growth inhibiting effects found in the cell extracts were artifacts produced during the extraction. In extracts of culture solutions, however, chlorophyllide was also found. Contrary to the chlorophyllide in the cell extracts the chlorophyllide found in the extract of the culture solution showed antibacterial effects without preceding illumination. This difference in the action of the chlorophyllide from cell extract and from extract of culture solution is of course due to the preceding illumination of the chlorophyllide in the culture solution. The chlorophyllide was already present when the algal culture was illuminated. These experiments were all carried out with cultures absolutely free of bacteria.

Most of the experiments reported in the present paper were carried out with algal cultures which were probably contaminated with bacteria. Hence we cannot say with certainty whether the substances produced in the algal cultures were produced by the algae, by the bacteria or by an interaction of the two kinds of organisms. The presence of bacteria may possibly stimulate the excretion of antibacterial substances from the algae. However, the experiments presented in Figures 2 and 3 were carried out with bacteria-free algal cultures. They show with certainty that both growth inhibiting and growth accelerating substances are found in bacteria-free cultures as well.

On the basis of our present knowledge of the substances produced by algae it is impossible to coordinate the results found by the various authors. The only thing which may be said with certainty is that the problems concerning the growth affecting substances in algal cultures are very complex.

The substances found in the algal cultures which accelerate the growth of *Staphylococcus* may possibly be some of the known vitamins. Algae are known to be able to synthesize vitamins. Thus the algae examined by Gerdes (1951) were found to contain thiamine. A content of vitamin B₁₂ was observed by Brown, Cuthbertson, and Fogg (1956) in extracted cells of *Chlorella vulgaris* and *Anabaena cylindrica*, and Coms (1952) has published analyses showing high levels of ten vitamins in *Chlorella pyrenoidosa*.

On the other hand, it was shown by Knight (1937) that thiamine is necessary for the growth of *Staphylococcus aureus* and experiments carried out by the present authors showed that vitamin B₁₂ stimulates the growth of *Staphylococcus* in concentrations of from 0.01 to 0.1 μg per ml medium.

Among the inhibitors found by different authors in algal culture solutions or in cell extracts of *Chlorella* some are influenced by illumination.

One of the inhibitors found in the experiments presented in Figure 6 was inactivated by illumination. One of the inhibitors found in cell extract by Blaauw-Jansen (1954) was similarly inactivated by illumination. Green light had no effect on the activity of the substance in question, while orange light decreases the activity strongly.

Other inhibitors found in *Chlorella* are on the contrary activated by illumination. The material extracted from *Chlorella* cells by Spoehr *et al.* (1949), in which the active components were suggested to be unsaturated fatty acids or related compounds, developed antibacterial properties when exposed to air and light.

The substance from cell extracts of *Chlorella* which had no relation to plant pigments (Jørgensen 1962) did show inhibiting effects in the dark, but the inhibition zone was extended by illumination.

It was stated by Blaauw-Jansen (1954) as well as by the junior author (Jørgensen 1962) that extracts of *Chlorella* cells contain chlorophyllide which by illumination is transformed into a growth inhibiting substance. As mentioned above Jørgensen (1962) further found that the examined culture solutions from cultures of *Chlorella vulgaris*, *Scenedesmus quadricauda* and *Chlamydomonas reinhardi* contained chlorophyllide. The chlorophyllide in the culture solution showed antibacterial activity without preceding illumination, since they had already been illuminated during the culturing of the algae.

The work in this laboratory on the production of antibacterial substances by plankton algae was originally designed with the idea in mind of finding

an explanation of some very striking observations made in experiments on the metabolism of oxygen in extremely unproductive ocean water.

If sea water is enclosed in bottles, bacteria at once start growing in great quantities. Due to the solid surfaces now present organic matter is at once attacked by the bacteria. It is present in concentrations of about 1.2–2.0 mg C per litre in all sea water, but cannot be attacked *in situ*. Due to the lack of solid surfaces the concentration is too low — cf. *e.g.* Zobell and Anderson (1936). If bottles with oceanic water are kept for, *e.g.*, three days in the dark, the oxygen consumption by these bacteria is easily measured. However, if the bottles are kept in the light, the oxygen consumption is very considerably lower. This decrease in the oxygen consumption was once considered to be due to photosynthesis (Riley 1939). However, experiments with the reliable carbon-14 technique show this explanation to be incorrect.

In some way or other light must be able to reduce the bacterial activity in bottles containing ocean water. Based on experimental data Steemann Nielsen 1955 concluded that the reduction was due to the production of an antibacterial substance by the algae. A direct influence of the light on the development of the bacteria cannot be ruled out, however — cf. Jørgensen and Steemann Nielsen 1960. The problems appear to be rather complex.

A number of facts support the hypothesis that chlorophyllide activated by light is a major factor. Most oceanic plankton algae perish rather rapidly if the water is kept in bottles. The cell walls are very thin and breakable. Chlorophyllide produced in the remains of the algae must thus come out into the water. In the illuminated bottles this chlorophyllide may be transformed into an antibacterial substance by photo-oxidation.

The problem may be still more complicated. It is a puzzling detail that occasionally the oxygen consumption in the dark is higher if the ocean water is filtered through filter paper which reduce the quantity of algae to about one quarter — cf. Steemann Nielsen 1958. This observation has been corroborated by Fogg 1958 in experiments of another kind. The dark fixation of carbon-14 in productivity experiments with the tracer technique was shown to increase by a factor 2.5 when 75 per cent of the lake water was filtered through a membrane filter. In this type of water dark fixation was even higher than the fixation in the light.

Interaction between phytoplankton and bacteria deserve — also as pointed out by Fogg — further investigation.

Summary.

Filtered culture solutions from cultures of different unicellular algae, especially *Chlorella vulgaris*, were tested for content of growth affecting

substances using the bacteria *Staphylococcus aureus* as test organism. Either a decrease or an increase of the growth of the bacteria could be found. It was observed that both growth accelerating and growth inhibiting substances were present simultaneously in the culture solution and that the concentration of the substances varies a great deal in cultures of the same algal species even if the cultures were grown under apparently identical conditions.

In one of the experiments the inhibiting effect was decreased by heating the algal filtrate at 70°C for 20 min.

In another experiment at least two inhibitors were found. One was inactivated by illumination of the algal filtrate, a second was absorbed to Norit.

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